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(54) Title: PLANT LYSOPHOSPHATIDIC ACID ACYLTRANSFERASES

(57) Abstract

This invention relates to plant LPAATs, means to identify such proteins, amino acid and nucleic acid sequences associated with such protein, and methods to obtain, make and/or use such plant LPAATs. Purification, especially the removal of plant membranes and the substantial separation away from other plant proteins, and use of the plant LPAAT is provided, including the use of the protein as a tool in gene isolation for biotechnological applications. In addition, nucleic acid sequences encoding LPAAT protein regions are provided, and uses of such sequences for isolation of LPAAT genes from plants and for modification of plant triglyceride compositions are described.

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PLANT LYSOPHOSPHATIDIC ACID ACYLTRANSFERASES

This application is a continuation-in-part of Application Serial No. 08/327,451 filed October 21, 1994, and a continuation-in-part of Application Serial No.08/254,404 filed June 6, 1994 and a continuation-in-part of Application Serial No.08/231,196 filed April 21, 1994 and a continuation-in-part of Application Serial No. 08/224,625 filed April 6, 1994.

Technical Field

The present invention is directed to protein preparations, amino acid and nucleic acid sequences and constructs, and methods related thereto.

INTRODUCTION

Background

There is a need for improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources. For example, novel oil products, improved sources of synthetic triacylglycerols (triglycerides), alternative sources of commercial oils, such as tropical oils (*i.e.*, palm kernel and coconut oils), and plant oils found in trace amounts from natural sources are desired for a variety of industrial and food uses.

To this end, the triacylglycerol (TAG) biosynthesis system in plants and bacteria has been studied. In the cytoplasmic membranes of plant seed tissues which accumulate storage triglycerides ("oil"), fatty acyl groups at the *sn*-2 position of the triglyceride molecules are incorporated *via* action of the enzyme 1-acylglycerol-3-phosphate acyltransferase (E.C. 2.3.1.51), also known as lysophosphatidic acid acyltransferase, or LPAAT.

By inspection of the LPAAT activities in isolated membranes from seed tissues, it has been shown that LPAAT specificities vary from species to species in accordance with the kinds of fatty acyl groups found in the *sn*-2

positions of the respective storage oils. For example, in the seeds of *Cuphea* species, which accumulate oils containing medium-chain fatty acids, it is possible to demonstrate an LPAAT activity which will utilize medium-chain acyl-CoA and lysophosphatidic acid (LPA) substrates. In contrast, LPAAT activity from the membranes of rapeseed embryos, in which the oil contains fatty acids of longer chain length, uses these medium-chain substrates much less readily, and predominantly uses long-chain unsaturated fatty acids. Similarly the meadowfoam plant (*Limnanthes alba*) accumulates an oil containing erucic acid (22:1) in all three *sn* positions and has a seed LPAAT activity able to use 22:1-CoA and 22:1-LPA, whereas rapeseed, which does not accumulate these fatty acids, has little or no such 22:1-utilizing LPAAT.

Similar studies with the enzymes responsible for the *sn*-1 and *sn*-3 acylations show that they are much less selective with respect to the substrate chain lengths. Thus, for a specific storage triglyceride in a given plant, the types of fatty acyl groups found in the *sn*-2 position of the oil are determined primarily by the specificity of LPAAT with respect to its acyl-donor substrates, *i.e.* acyl-CoAs. In addition, the selectivity of the LPAAT towards the acyl-CoAs is also influenced by the nature of the acyl group already attached in the *sn*-1 position of the acceptor substrates, *i.e.* the 1-acylglycerol-3-phosphate or lysophosphatidic acid (LPA) molecules.

The characterization of lysophosphatidic acid acyltransferase (also known as LPAAT) is useful for the further study of plant FAS systems and for the development of novel and/or alternative oils sources. Studies of plant mechanisms may provide means to further enhance, control, modify or otherwise alter the total fatty acyl composition of triglycerides and oils. Furthermore, the elucidation of the factor(s) critical to the natural production of triglycerides in plants is desired, including the purification of such factors and the characterization of element(s) and/or co-factors which enhance the efficiency of the system. Of special interest are the nucleic acid

sequences of genes encoding proteins which may be useful for applications in genetic engineering.

Literature

Published characterizations of acyltransferase specificities in rapeseed membranes report that acyl group discrimination occurs primarily at the *sn*-2 acylation (Oo *et al.*, *Plant Physiol.* (1989) *91*:1288-1295; Bernerth *et al*, *Plant Sci.* (1990) *67*:21-28).

Coleman (*Mol. Gen. Genet.* (1992) *232*:295-303) reports the characterization of an *E. coli* gene (*plsC*) encoding LPAAT. The *E. coli* LPAAT is capable of utilizing either acyl-CoA or acyl-ACP as the fatty acyl donor substrate.

Hares & Frentzen (*Planta* (1991) *185*:124-131) report solubilization and partial purification of a long-chain preferring LPAAT from endoplasmic reticulum in pea shoots. The purported solubilization is based solely on the inability to sediment LPAAT activity by high-speed centrifugation.

Wolter *et al.* (*Fat Sci. Technol.* (1991) *93*: 288-290) report failed attempts to purify a *Limnanthes douglasii* acyltransferase catalyzing the acylation of erucic acid to the *sn*-2 position of the glycerol backbone, and propose hypothetical methods of gene isolation based on cDNA expression in microorganisms.

Nagiec *et al.* (*J. Biol. Chem.* (1993) *268*:22156-22163) report the cloning of an *SLCI* (sphingolipid compensation) gene from yeast and report homology of the encoded protein to the LPAAT protein of *E. coli*.

Taylor *et al.* (in "Seed Oils for the Future", ed. Mackenzie & Taylor (1992) AOCS Press) report acyl-specificities for 18:1-CoA and 22:1-CoA substrates for LPAATs from several plant species and discuss attempts to purify a *B. napus* LPAAT.

Slabas *et al.* (Ch. 5, pages 81-95 (1993) in *Seed Storage Compounds: Biosynthesis, Interactions, and Manipulation*, ed Shewry & Stobart, Clarendon Press) discuss attempts to purify plant LPAAT proteins and note that all attempts to purify LPAAT to homogeneity have failed.

Attempts to clone a corn LPAAT gene by complementation of an *E. coli* mutation at *plsC* are also discussed.

Oo *et al.* (*Plant Physiol.* (1989) *91*:1288-1295) report characterization of LPAAT specificities in membrane preparations of palm endosperm, maize scutellum, and rapeseed cotyledon.

Cao *et al.* (*Plant Physiol.* (1990) *94*:1199-1206) report characterization of LPAAT activity in maturing seeds of meadowfoam, nasturtium, palm, castor, soybean, maize, and rapeseed. LPAAT activity was characterized with respect to 22:1 and 18:1 LPA and acyl-COA substrates.

Laurent and Huang (*Plant Physiol.* (1992) *99*:1711-1715) report that LPAATs in palm and meadowfoam which are capable of transferring 12:0 and and 22:1 acyl-CoA substrates to the *sn*-2 position of LPA, are confined to the oil-accumulating seed tissues.

Bafor *et al.* (*Phytochemistry* (1990) *31*:2973-2976) report substrate specificities of TAG biosynthesis enzymes, including LPAAT, from *Cuphea procumbens* and *C. wrighti*.

Bafor *et al.* (*Biochem. J.* (1990) *272*:31-38) report results of studies on regulation of TAG biosynthesis in *Cuphea lanceolata* embryos. Results of assays for LPAAT activity in microsomal preparations from developing cotyledons are provided.

Frentzen *et al.* (*Eur. J. Biochem.* (1990) *187*:389-402 report characterization of mitochondrial LPAAT activity in potato tubers and pea leaves.

Hanke & Frentzen at Congress on Plant Lipids, Paris, July 1, 1994 reported the obtention of a meadowfoam 1030 bp clone encoding a potential protein of 31 kDa. No sequence was shown but they indicated a "substantial" similarity to *E. coli plsC* and that this match was better than putative yeast LPAAT. The clone was reportedly obtained from a developing seed cDNA library in complementation studies with an *E. coli* LPAAT mutant. It was also reported that their clone demonstrated a higher preference for 22:1 CoA than 18:1 CoA as the acyl donor and that northern analysis showed expression in meadowfoam embryo and not in leaves.

Brown & Slabas, at the 4th International Congress of Plant Molecular Biology, Amsterdam, June 19, 1994, showed a partial amino acid sequence reported to be a maize LPAAT obtained using a maize embryo culture cDNA to complement the *E. coli* LPAAT mutation. The molecular weight of the protein was reported at about 45 kDa with homologies to *E. coli plsC* and the yeast AT. Also, see WO94/13814, published June 23, 1994, which gives a sequence identified as the cDNA sequence of maize 2-acyltransferase.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the effect of soybean phospholipid concentration on coconut medium-chain LPAAT activity (assay of S3 preparation).

Figure 2 shows the results of chromatography of bay P2 preparation on Sephacryl S400 column.

Figure 3 shows the results of a bay supernatant fraction prepared according to Frentzen *et al.*, and chromatographed on a Sephacryl S400 column.

Figure 4 shows the results of chromatography of the bay S3 preparation on a Superose 6 column.

Figure 5 provides a demonstration of the effects of solubilization by CHAPS concentration and detergent/protein (D/P) ratio, as measured by the yield of coconut medium-chain LPAAT activity in the S3 preparation.

Figure 6 shows the chromatography of coconut S3 preparation on red 120 agarose.

Figure 7 shows the results of chromatography of coconut medium-chain LPAAT activity from the red 120 column on a column of hydroxyapatite.

Figure 8 shows the results of partially purified coconut medium-chain LPAAT preparation passed through a 12:0-CoA chromatography column.

Figure 9 provides the results of chromatography of partially purified, PL-activated coconut medium-chain LPAAT preparation on a 12:0-CoA column in the presence of phospholipids.

Figure 10 provides DNA sequence and translated amino acid sequence of a clone, 23-2, containing coconut LPAAT encoding sequence obtained by PCR.

Figure 11 provides DNA sequence and translated amino acid sequence of a clone, 23-4, containing coconut LPAAT encoding sequence obtained by PCR.

Figure 12 provides DNA sequence and translated amino acid sequence of a clone, 10-1, containing coconut LPAAT encoding sequence obtained by PCR.

Figure 13 provides DNA sequence and translated amino acid sequence of full length coconut LPAAT clone COLP4 (pCGN5503).

Figure 14 provides DNA sequence and translated amino acid sequences of a clone, MeadLPAAT 15, containing meadowfoam LPAAT encoding sequence obtained by PCR.

Figure 15 provides DNA sequence and translated amino acid sequences of a clone, MeadLPAAT 20, containing meadowfoam LPAAT encoding sequence obtained by PCR.

Figure 16 shows a comparison of translated amino acid sequences of clones COLP4, MeadLPAAT 15 and MeadLPAAT 20.

Figure 17 provides DNA sequence and translated amino acid sequences of meadowfoam LPAAT cDNA clone Melp2.

Figure 18 provides DNA sequence and translated amino acid sequences of meadowfoam LPAAT cDNA clone Melp4.

SUMMARY OF THE INVENTION

This invention relates to plant proteins which catalyze the production of 1,2-diacylglycerol-3-phosphate from 1-acylglycerol-3-phosphate (also referred to as lysophosphatidic acid or LPA) and an acyl-CoA substrate. Such proteins are referred to herein as 1-acylglycerol-3-phosphate acyltransferases (E.C. 2.3.1.51) or LPAATs. In particular, the LPAAT proteins of this invention demonstrate preferential activity on acyl-CoA donor substrates and little or no activity towards acyl-ACP donor substrates.

By this invention, a new class of plant LPAAT proteins which have been substantially purified away from the

cytoplasmic membranes of their native plant host may now be characterized with respect to preferential substrate activity. In particular, purification of a plant LPAAT enzyme having preferential activity towards medium-chain acyl-CoA substrates is provided.

A medium-chain preferring LPAAT of this invention demonstrates a preference for medium-chain acyl-CoA donor substrates, whether the LPA acceptor substrate contains a medium-chain acyl group (such as C12:0) at the *sn*-1 position or a long-chain acyl group (such as C18:1) at the *sn*-1 position. A coconut endosperm medium-chain acyl-CoA preferring LPAAT enzyme is exemplified herein. Lauroyl-CoA is a preferred donor substrate when the acceptor substrate is either 1-lauroylglycerol-3-phosphate or 1-oleoylglycerol-3-phosphate. In addition, the coconut LPAAT also demonstrates preferential activity on other medium-chain acyl-CoA substrates, particularly those having C10 or C14 carbon chains, as compared to longer chain length (C16 or C18) substrates.

The exemplified coconut LPAAT is purified away from the membranes (*i.e.* solubilized), and the solubilized LPAAT preparation is subjected to various chromatographic analyses to identify a protein associated with the LPAAT activity. In this manner a protein having a molecular weight of approximately 27-29kDa is identified as associated with LPAAT activity. Further purification methods, such as column chromatography and polyacrylamide gel electrophoresis, are utilized to obtain the LPAAT protein in sufficient purity for amino acid sequence analysis.

As a result, LPAAT peptide sequences are determined, and an LPAAT peptide fragment having sequence homology to non-plant LPAATs (*E. coli plsC* gene product and a putative yeast AT) is discovered. The LPAAT peptide sequences are used as templates in designing various synthetic oligonucleotides which are then used to obtain nucleic acid sequences encoding all or a portion of the coconut LPAAT protein.

LPAAT PCR product sequences are provided in the instant application and used to obtain cDNA clones encoding coconut LPAAT, sequence of which is also provided herein. Using the coconut LPAAT encoding sequences so obtained, it is also possible to isolate other plant LPAAT genes which encode LPAAT proteins of different specificities with respect to acyl-CoA donor substrates (*e.g.* 8:0, 10:0, 14:0, 22:1 etc.). For example, using the coconut sequence, sequence of meadowfoam LPAAT clones are now provided. A comparison of the coconut and meadowfoam LPAAT sequences provides additional conserved amino acid sequences which are further useful to the identification of LPAAT genes from other sources.

Thus, this invention encompasses plant LPAAT peptides and the corresponding amino acid sequences of those peptides, and the use of these peptide sequences in the preparation of oligonucleotides containing LPAAT encoding sequences for analysis and recovery of plant and non-plant LPAAT gene sequences. The plant LPAAT encoding sequence may encode a complete or partial sequence depending upon the intended use. All or a portion of the genomic sequence, or cDNA sequence, is intended.

Of special interest are recombinant DNA constructs which provide for transcription or transcription and translation (expression) of the plant LPAAT sequences. In particular, constructs which are capable of transcription or transcription and translation in plant host cells are preferred. For some applications a reduction in plant LPAAT may be desired. Thus, recombinant constructs may be designed having the plant LPAAT sequences in a reverse orientation for expression of an anti-sense sequence or use of co-suppression, also known as "transwitch", constructs may be useful. Such constructs may contain a variety of regulatory regions including transcriptional initiation regions obtained from genes preferentially expressed in plant seed tissue. For some uses, it may be desired to use the transcriptional and translational initiation regions of the LPAAT gene either with the LPAAT encoding sequence or

to direct the transcription and translation of a heterologous sequence.

In yet a different aspect, this invention relates to a method for producing a plant LPAAT in a host cell or progeny thereof via the expression of a construct in the cell. Cells containing a plant LPAAT as a result of the production of the plant LPAAT encoding sequence are also contemplated herein.

In addition, this invention relates to methods of using DNA sequences encoding plant LPAAT for the modification of the composition of fatty acyl groups at the *sn*-2 position of the triglyceride molecules, especially in the seed oil of plant oilseed crops. Plant cells having such a modified triglyceride are also contemplated herein. Of particular interest is the use of a medium-chain preferring LPAAT sequence in *Brassica* plants which have been engineered to produce medium-chain fatty acids in the seed oil. In such plants, up to approximately 50 mol percent laurate is accumulated in the seed triglycerides. Most of this laurate, however, is esterified at the *sn*-1 and *sn*-3 positions due to the specificity of the *Brassica* LPAAT for longer chain length acyl-CoA substrates. By expression of a medium-chain preferring LPAAT protein in the seeds of such plants, it is possible to obtain *Brassica* seed oil which has greater than 67 mole percent laurate in the TAG.

Also of particular interest is the production of tri-erucin in high erucic acid plants such as high erucic acid rapeseed (HEAR) oil varieties or to further decrease erucic acid composition of plants containing erucic fatty acids in the *sn*-2 position of a plant TAG. For example, by expression of a very long-chain preferring LPAAT protein in the seeds of HEAR oil varieties, it is possible to obtain *Brassica* seed oil which has a greater than 66 mole percent erucin in the TAG.

Also considered in this invention are the modified plants, seeds and oils obtained by expression of the plant LPAAT sequences and proteins of this invention.

DETAILED DESCRIPTION OF THE INVENTION

A plant LPAAT of this invention includes any sequence of amino acids, such as a protein, polypeptide or peptide, obtainable from a plant source, which demonstrates the ability to catalyze the production of 1,2-diacylglycerol-3-phosphate from 1-acylglycerol-3-phosphate and an acyl-CoA substrate under plant enzyme reactive conditions. By "enzyme reactive conditions" is meant that any necessary conditions are available in an environment (i.e., such factors as temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

Preferential activity of a plant LPAAT toward particular chain-length fatty acyl-CoA substrates is determined upon comparison of 1,2-diacylglycerol-3-phosphate product amounts obtained per different chain length acyl-CoA donor substrates. In some cases, the chain length of an acyl group in the *sn*-1 position may also affect the ability of the LPAAT to utilize a given chain length acyl-CoA donor. Of particular interest in the instant invention is a medium-chain acyl-CoA preferring LPAAT in coconut immature endosperm tissue and a very long-chain acyl-CoA preferring LPAAT active in developing meadowfoam embryo tissue.

By medium-chain acyl-CoA preferring is meant that the enzyme preparation demonstrates a preference for medium-chain, i.e. C8, C10, C12 or C14 acyl-CoA donor substrates over acyl-CoA substrates of different acyl carbon lengths, regardless of the chain length of the acyl group in the *sn*-1 position of the acceptor LPA substrate. By long-chain acyl- CoA is meant that the enzyme preparation demonstrates a preference for long-chain, i.e., C16 and C18, donor substrates over acyl-CoA substrates of different acyl carbon lengths. And in a similar fashion, very-long-chain acyl-CoA preferring LPAAT will demonstrate a preference for a very long chain, i.e., C20, C22 and greater, donor substrates. It is noted that some activity, of a lesser magnitude, may also be observed against other chain-length fatty acyl substrates, i.e., the specificity will be substantial, but may not be absolute. For example, the

exemplified coconut LPAAT demonstrates a strong preference for C12 acyl-CoA donor substrates when the acceptor substrate is lauroyl-LPA, but also has significantly more activity towards C10 and C14 substrates as compared to longer chain substrates whose acyl groups have 16 or 18 carbons. When the acceptor substrate is 18:1-LPA, the coconut LPAAT uses C12 and C14 substrates at nearly equal rates, and still prefers these and C10 substrates over available longer chain acyl-CoA substrates.

Other plant LPAAT proteins may also demonstrate preferential activity on one or more medium-chain, long-chain or very-long-chain acyl-CoA substrates, but the preference may only be encountered where a particular, e.g. medium-chain, acyl group is present in the *sn*-1 position of the LPA donor substrate. Such LPAATs are considered as having selective preference for such substrate.

As noted above, a plant LPAAT of this invention will display activity toward fatty acyl-CoA substrates, and have little or no activity towards fatty acyl-ACP substrates. Thus, the LPAAT of the instant invention may be distinguished from plant chloroplastic LPAATs which demonstrate activity towards both acyl-ACP and acyl-CoA substrates.

The acyl-CoA LPAATs of the instant invention are present in cytoplasmic membranes in various plant tissues. Of particular interest are those LPAATs associated with the TAG biosynthesis pathway in the endoplasmic reticulum of immature seed tissues. Immature seed tissues containing such LPAATs may include embryo tissue or endosperm tissue, depending on the location of TAG biosynthesis in a particular plant species. In coconuts, for example, LPAAT activity is detected primarily in the endosperm tissue, the site of TAG biosynthesis. In California bay seeds, immature embryo cotyledons provide a good source of LPAAT activity, and in *Brassica* seeds, substantial LPAAT activity is also found in immature embryos. In meadowfoam plants, LPAAT activity is found in immature embryos.

The plant endoplasmic reticulum LPAAT enzymes studied to date have been found to be membrane proteins. Thus, in

order to further study LPAAT activity, and in particular to produce purified preparations of such a protein by chromatographic methods, it is necessary to obtain the enzyme in solubilized form, i.e. separated from the cytoplasmic membrane environment.

"Solubilization" refers to extraction of the LPAAT enzyme from the membranes in such a way that it then behaves in a manner typical of enzymes that are not membrane-associated. Because the membrane effectively links the LPAAT protein to other proteins which are also present therein, solubilization is an essential requirement for identification and purification of the LPAAT protein as described in the following examples. In testing for solubilization of LPAAT activity, three different indications of solubilization, as described in more detail in the following examples, are considered.

1) LPAAT activity is not sedimented by very high-speed centrifugation.
2) LPAAT activity migrates on a size-exclusion chromatography column as though it had a native molecular weight typical of enzymes which are not membrane-associated.
3) Proteins present in the LPAAT preparation are at least partially separable from each other by column chromatography.

Because of potential alternative interpretations that may apply to any of the above criteria individually, it is necessary to confirm that all three of the criteria have been satisfied to confirm LPAAT solubilization. For example, the first criterion, of failure to sediment at very high *g* forces could be misleading if the density of the solution used for solubilization is similar to that of the unsolubilized membranes so that they sediment only very slowly. This situation is illustrated in the examples which follow, in which a published solubilization procedure that relied on this criterion alone is shown to be inadequate to obtain LPAAT substantially separated from the

cytoplasmic membranes. The second criterion, in which solubilized activity migrates more slowly through a size-exclusion column than the original membranes, may be compromised if the membranes themselves bind weakly to the column after exposure to detergent so that their migration through it is slowed. The third criterion, in which the solubilized proteins are chromatographically resolvable, is the least likely to be compromised by artifacts or unforeseen situations. However, it is possible that membranes could be partially dissociated by the solubilization procedure such that various aggregates of proteins are released. Such aggregates might then be resolved from each other chromatographically. Thus, satisfaction of all three criteria is necessary to assure that LPAAT solubilization is achieved.

Solubilization of coconut LPAAT in a solution containing 1M NaCl, 2.25% (w/v) CHAPS detergent, and a detergent/protein ratio of 48/1 (w/w) is described in the following examples. Similarly, LPAAT activity from California bay is solubilized using a solubilization solution containing 1M NaCl, 4% (w/v) CHAPS detergent, and a detergent/protein ratio of 58/1 (w/w) Solubilization of the plant LPAATs is confirmed by demonstration of each of the above criteria of solubilization.

Furthermore, in studies of the solubilized LPAAT activity it was discovered, as described in detail in the following examples, that solubilized LPAAT could only be assayed by addition of concentrated phospholipids, to reconstitute LPAAT activity. In particular, the stimulatory action of phospholipds on LPAAT activity is greatest when the phospholipids are added to the solubilized LPAAT sample at the start of the assay procedure, followed by dilution of the high CHAPS and salt concentrations in this buffer by addition of the remaining assay ingredients. Addition of the phospholipids after dilution of the solubilization solution results in little or no increase in detection of LPAAT activity. The phospholipid stimulation effect is also seen where the phospholipids are added to a sample of solubilization

buffer alone, followed by dilution with remaining assay ingredients and subsequent addition of the solubilized LPAAT sample.

Solubilized preparations of coconut endosperm LPAAT are utilized in a variety of chromatographic experiments for identification and partial purification of the LPAAT protein. In this manner, a protein having a molecular weight of approximately 27-29kDa is identified as associated with LPAAT activity. As described in more detail in the following examples, the 29kDa protein is partially purified by chromatography on red 120 agarose and hydroxyapatite columns. The protein is then obtained in substantially purified form by gel electrophoresis and blotting of the partially purified LPAAT preparation to nitrocellulose. The 27-29kDA protein is recovered by cutting out that portion of the nitrocellulose filter containing the identified band.

The purified protein is then digested with various enzymes to generate peptides for use in determination of amino acid sequence. Amino acid sequence of a tryptic peptide obtained in this manner is demonstrated to share a region of homology with the LPAAT protein encoded by the *E. coli plsC* gene. This same region shared by the *E. coli* and coconut LPAATs is also found in a yeast acyltransferase protein encoded by the *SLC1* gene.

Thus, the tryptic peptide of the 27-29kDa protein described herein represents a portion of a medium chain-acyl-CoA preferring coconut LPAAT. Other coconut LPAAT peptides are similarly obtained and the amino acid sequences provided.

The use of amino acid sequences from LPAAT peptides to obtain nucleic acid sequences which encode coconut or other LPAAT genes is described herein. For example, synthetic oligonucleotides are prepared which correspond to the LPAAT peptide sequences. The oligonucleotides are used as primers in polymerase chain reaction (PCR) techniques to obtain partial DNA sequence of LPAAT genes. The partial sequences so obtained are then used as probes to obtain LPAAT clones from a gene library prepared from coconut or

other tissue of interest. As an alternative, where oligonucleotides of low degeneracy can be prepared from particular LPAAT peptides, such probes may be used directly to screen gene libraries for LPAAT gene sequences. In particular, screening of cDNA libraries in phage vectors is useful in such methods due to lower levels of background hybridization. DNA sequences of LPAAT peptide encoding sequences obtained in this manner are provided in the application.

A nucleic acid sequence of a plant or other LPAAT of this invention may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or in part. The gene sequences may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR).

Alternatively, the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene (that portion of the gene which encodes the LPAAT protein) may be synthesized using codons preferred by a selected host. Host-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" LPAATs from a variety of plant and other sources. Homologous sequences are found when there is an identity of sequence, which may be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known LPAAT and a candidate source. Conservative changes, such as Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may also be considered in determining sequence homology. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature

proteins. (*See* generally, Doolittle, R.F., *OF URFS and ORFS* (University Science Books, CA, 1986.)

Thus, other plant LPAATs may be obtained from the specific exemplified coconut protein preparations and sequences provided herein, such as the meadowfoam LPAAT described herein. The meadowfoam LPAAT sequence, which is from a dicotyledonous plant and the coconut LPAAT sequence, which is from a monocotyledonous plant, may be used to identify highly conserved amino acid sequences representative of LPAATs in the plant kingdom. Such regions include the peptides: LLPWPY, GNLYGH, RIDRSNP, KNLSLI, LPIVPM, FPEGTRS, GRLLPFKKGF, LTGTHLAWRK and PITVKY. Using degenerate oligonucleotides that encode these sequences and PCR techniques, the LPAAT from any plant species and particularly any cytoplasmic acyl-CoA active and acyl-ACP inactive LPAATs, may be obtained.

In addition, it is now found that LPAATs from *E. coli*, coconut and meadowfoam have regions of conserved amino acid sequence, which regions are also conserved in a putative LPAAT protein from yeast. Thus, it may be possible to design probes from such conserved regions to isolate LPAAT encoding sequences from other organisms, such as from animals. Such LPAAT encoding sequences may also find use in applications described herein, in particular, in plant genetic engineering techniques for production of TAG having particular fatty acyl groups at the *sn*-2 position. For example, an animal LPAAT may find applications in plant genetic engineering to produce oils having long-chain saturated fatty acyl groups, such as 18:0 in the *sn*-2 position to provide a source of useful TAG for infant formula.

Furthermore, it will be apparent that one can obtain natural and synthetic LPAATs, including modified amino acid sequences and starting materials for synthetic-protein modeling from the exemplified plant LPAATs and from LPAATs which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences which have been mutated, truncated, increased and the like, whether such sequences were partially or wholly

synthesized. Sequences which are actually purified from plant preparations or are identical or encode identical proteins thereto, regardless of the method used to obtain the protein or sequence, are equally considered naturally derived.

Typically, an LPAAT sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target LPAAT sequence and the encoding sequence used as a probe. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probe may be a lengthy fragment of the nucleic acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100 bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80% sequence identity) from the sequences used as probe. Oligonucleotide probes can be considerably shorter than the entire nucleic acid sequence encoding an LPAAT enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify regions of highly conserved amino acid sequence to design oligonucleotide probes for detecting and recovering other related LPAAT genes. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (*See*, Gould, *et al.*, *PNAS USA* (1989) *86*:1934-1938.)

In addition to isolation of other plant LPAATs, it is considered that genes for other related acyltransferase proteins may also be obtained using sequence information from the coconut LPAAT and related nucleic acid sequences. For example, other acyltransferase enzymes are involved in plant lipid biosynthesis, including plastidial LPAAT, mitochondrial LPAAT, lysophosphosphatidylcholine acyltransferase (LPCAT), lysophosphosphatidylserine acyltransferase (LPSAT), lysophosphosphatidylethanolamine

acyltransferase (LPEAT), and lysophosphosphatidylinositol acyltransferase (LPIAT). These enzymes all catalyze acyltransferase reactions involving the *sn*-2 position of lysophospholipids, and the genes encoding these sequences may also be related to the plant acyl-CoA LPAAT sequences of the instant invention and obtainable therefrom.

To determine if a related gene may be isolated by hybridization with a given sequence, the sequence is labeled to allow detection, typically using radioactivity, although other methods are available. The labeled probe is added to a hybridization solution, and incubated with filters containing the desired nucleic acids, such as Northern or Southern blots, or the filters containing cDNA or genomic clones to be screened. Hybridization and washing conditions may be varied to optimize the hybridization of the probe to the sequences of interest. Lower temperatures and higher salt concentrations allow for hybridization of more distantly related sequences (low stringency). If background hybridization is a problem under low stringency conditions, the temperature can be raised either in the hybridization or washing steps and/or salt content lowered to improve detection of the specific hybridizing sequence. Hybridization and washing temperatures can be adjusted based on the estimated melting temperature of the probe as discussed in Beltz, *et al.* (*Methods in Enzymology* (1983) *100*:266-285). In particular, such screening methods may be used to screen mRNA preparations from seed tissues of a variety of plant species to identify related LPAAT or other acyl transferase genes which may be isolated using LPAAT gene sequences as probes. A useful probe and appropriate hybridization and washing conditions having been identified as described above, cDNA or genomic libraries are screened using the labeled sequences and additional plant LPAAT genes are obtained. One technique found useful in PCR, the amplification of the meadowfoam LPAAT, when a combination of coconut primers was used, was to denature the DNA and lower the temperature rapidly to about 65° C and then

slowly lower the temperature to the annealing temperature (40°- 50°C).

For immunological screening, antibodies to the coconut LPAAT protein can be prepared by injecting rabbits or mice with the purified protein, such methods of preparing antibodies being well known to those in the art. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation. Western analysis may be conducted to determine that a related protein is present in a crude extract of the desired plant species, as determined by cross-reaction with the antibodies to the coconut LPAAT. When cross-reactivity is observed, genes encoding the related proteins are isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Maniatis, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

All plants utilize LPAAT proteins in production of membrane phospholipids, and thus any given plant species can be considered as a source of additional LPAAT proteins. Plants having significant medium-chain fatty acids in their seed oils are preferred candidates to obtain plant LPAATs capable of incorporating medium-chain fatty acids into the *sn*-2 position of TAG. Several species in the genus *Cuphea* accumulate triglycerides containing medium-chain fatty acids in their seeds, e.g., *procumbens, lutea, hookeriana, hyssopifolia, wrightii* and *inflata*. Another natural plant source of medium-chain fatty acids are seeds of the *Lauraceae* family. In addition to the exemplified California Bay (*Umbellularia californica*), Pisa (*Actinodophne hookeri*), Sweet Bay (*Laurus nobilis*) and *Cinnamomum camphora* (camphor) accumulate medium-chain fatty acids. Other plant sources include *Ulmaceae* (elm), *Palmae, Myristicaceae, Simarubaceae, Vochysiaceae*, and *Salvadoraceae*.

Also of particular interest are LPAATs from plant species which incorporate unusual longer-chain fatty acids in the storage TAG. For example nasturtium and meadowfoam contain 22:1 acyl groups in the seed TAG, and meadowfoam has been shown to contain an LPAAT capable of incorporating 22:1 (erucic) fatty acyl groups into the *sn*-2 position. An LPAAT having such activity may find use in production of "tri-erucic" *Brassica* oil, which to date is not found due to the selectivity of *Brassica* seed LPAAT towards unsaturated fatty acids, such as 18:1 and 18:2. In fact, analysis of the triglycerides show that 22:1 is excluded from the *sn*-2 position of the triglycerides. This limits the theoretical maximum erucic acid content of rapeseed oil to 66 mole percent.

In addition, LPAAT enzymes from plants which contain other unusual fatty acids are of interest and may find use for production of TAG containing these unusual fatty acids in various plant species. Of interest in this regard are LPAATs involved in the production of acetylenic fatty acids, such as crepenynic acid from *Crepis foetida*; fatty acids with cyclopentene substituents, such as gorlic acid from species of the family *Flacourtiaceae*; cyclopropane fatty acids, such as vernolic acid from *Vernonia galamensis*; hydroxylated fatty acids, such as ricinoleic acid from *Ricinus communis*; furan-containing fatty acids, such as from *Exocarpus cupressiformis*; fatty acids with several unusual functional groups, such as those from *Sapium sebiferum*, which contain multiple double bonds and an internal ester function; fatty acids with unusual double-bond placement, such as petroselinic acid from some species of *Umbelliferae*, *Araliaceae*, and *Garryaceae*; and medium-chain fatty acids containing double bonds, such as from *Lindera* species.

It should also be noted that plant LPAATs from a variety of sources can be used to investigate TAG biosynthesis events of plant lipid biosynthesis in a wide variety of *in vivo* applications. Because all plants appear to synthesize lipids via a common metabolic pathway, the study and/or application of one plant LPAAT to a

heterologous plant host may be readily achieved in a variety of species. In other applications, a plant LPAAT can be used outside the native plant source of the LPAAT to enhance the production and/or modify the composition of the TAG produced or synthesized *in vitro*.

The nucleic acid sequences associated with plant or other LPAAT proteins will find many uses. For example, recombinant constructs can be prepared which can be used as probes, or which will provide for expression of the LPAAT protein in host cells to produce a ready source of the enzyme and/or to modify the composition of triglycerides found therein. Other useful applications may be found when the host cell is a plant host cell, either *in vitro* or *in vivo*. For example, by increasing the amount of a respective medium-chain or very-long-chain preferring LPAAT available to the plant TAG biosynthesis pathway, an increased percentage of medium-chain fatty acids or very-long-chain fatty acids, respectively, may be obtained in the TAG. In a like manner, for some applications it may be desired to decrease the amount of LPAAT endogenously expressed in a plant cell by anti-sense technology. For example, to allow for more opportunity for an inserted foreign LPAAT to transfer medium-chain or unusual longer-chain fatty acyl groups to the *sn*-2 position, decreased expression of a native *Brassica* long-chain preferring LPAAT may be desired.

Thus, depending upon the intended use, the constructs may contain the sequence which encodes the entire LPAAT protein, or a portion thereof. For example, where antisense inhibition of a given LPAAT protein is desired, the entire LPAAT sequence is not required. Furthermore, where LPAAT constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of an LPAAT encoding sequence, for example a sequence which is discovered to encode a highly conserved LPAAT region.

As discussed above, nucleic acid sequence encoding a plant or other LPAAT of this invention may include genomic, cDNA or mRNA sequence. By "encoding" is meant that the

sequence corresponds to a particular amino acid sequence either in a sense or anti-sense orientation. By "extrachromosomal" is meant that the sequence is outside of the plant genome of which it is naturally associated. By "recombinant" is meant that the sequence contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like.

A cDNA sequence may or may not contain pre-processing sequences, such as transit peptide sequences or targeting sequences to facilitate delivery of the LPAAT protein (such as mitochondrial LPAAT) to a given organelle or membrane location. The use of any such precursor LPAAT DNA sequences is preferred for uses in plant cell expression. A genomic LPAAT sequence may contain the transcription and translation initiation regions, introns, and/or transcript termination regions of the plant LPAAT, which sequences may be used in a variety of DNA constructs, with or without the LPAAT structural gene. Thus, nucleic acid sequences corresponding to the plant LPAAT of this invention may also provide signal sequences useful to direct protein delivery into a particular organellar or membrane location, 5' upstream non-coding regulatory regions (promoters) having useful tissue and timing profiles, 3' downstream non-coding regulatory regions useful as transcriptional and translational regulatory regions, and may lend insight into other features of the gene.

Once the desired plant or other LPAAT nucleic acid sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized. In the structural gene, one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be

further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

The nucleic acid or amino acid sequences encoding a plant or other LPAAT of this invention may be combined with other non-native, or "heterologous", sequences in a variety of ways. By "heterologous" sequences is meant any sequence which is not naturally found joined to the native (or wild-type) LPAAT, including, for example, combinations of nucleic acid sequences from the same plant which are not naturally found joined together.

The DNA sequence encoding a plant or other LPAAT of this invention may be employed in conjunction with all or part of the gene sequences normally associated with the LPAAT. In its component parts, a DNA sequence encoding LPAAT is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription and translation in a host cell, the DNA sequence encoding plant LPAAT and a transcription and translation termination region.

Potential host cells include both prokaryotic and eukaryotic cells. A host cell may be unicellular or found in a multicellar differentiated or undifferentiated organism depending upon the intended use. Cells of this invention may be distinguished by having an LPAAT foreign to the wild-type cell present therein, for example, by having a recombinant nucleic acid construct encoding a plant LPAAT therein not native to the host species.

Depending upon the host, the regulatory regions will vary, including regions from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Expression in a microorganism can provide a ready source of the plant enzyme. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Sacchromyces cerevisiae*, including genes such as

beta-galactosidase, T7 polymerase, tryptophan E and the like.

In a preferred embodiment, the constructs will involve regulatory regions functional in plants which provide for modified production of plant LPAAT, and, possibly, modification of the fatty acid composition. The open reading frame coding for the plant LPAAT or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region. In embodiments wherein the expression of the LPAAT protein is desired in a plant host, the use of all or part of the complete plant LPAAT gene is desired; namely all or part of the 5' upstream non-coding regions (promoter) together with the structural gene sequence and 3' downstream non-coding regions may be employed.

If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source, numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the structural gene functions. The transcription/translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons. Among transcriptional initiation regions used for plants are such regions associated with the T-DNA structural genes such as for nopaline and mannopine synthases, the 19S and 35S promoters from CaMV, and the 5' upstream regions from other plant genes such as napin, ACP, SSU, PG, zein, phaseolin E, and the like. Enhanced promoters, such as double 35S, are also available for expression of LPAAT sequences. For such applications when 5' upstream non-coding regions are obtained from other genes regulated during seed maturation, those preferentially expressed in plant embryo tissue, such as ACP and napin-derived transcription initiation control regions, are desired. Such "seed-specific promoters" may be obtained and used in

accordance with the teachings of U.S. Serial No. 07/147,781, filed 1/25/88 (now U.S. Serial No. 07/550,804, filed 7/9/90), and U.S. Serial No. 07/494,722 filed on or about March 16, 1990 having a title "Novel Sequences Preferentially Expressed In Early Seed Development and Methods Related Thereto," which references are hereby incorporated by reference. Transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts, are considered desirable for TAG modifications in order to minimize any disruptive or adverse effects of the gene product.

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the plant LPAAT or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. Where the transcript termination region is from a different gene source, it will contain at least about 0.5 kb, preferably about 1-3 kb of sequence 3' to the structural gene from which the termination region is derived.

Plant expression or transcription constructs having a plant LPAAT as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Most especially preferred are temperate oilseed crops. Plants of interest include, but are not limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledenous and monocotyledenous species alike and will be readily

applicable to new and/or improved transformation and regulation techniques.

Of particular interest is the use of plant LPAAT constructs in plants which have been genetically engineered to produce a particular fatty acid in the plant seed oil, where TAG in the seeds of nonengineered plants of the engineered species do not naturally contain that particular fatty acid. For example, in *Brassica* plants which have been genetically engineered to produce the medium-chain fatty acids, and in particular laurate (12:0), in the seed oil, a deficiency in *sn*-2 acylation has been discovered. (See WO 92/20236.) For example, in oil from plants in which 40% of the seed oil fatty acyl groups have been changed from the long-chain (primarily 18:1) type to 12:0, the 12:0 enrichment at the *sn*-1 *and* *sn*-3 positions (averaged together) is approximately 50% and the 12:0-enrichment at the *sn*-2 position is approximately 12%. Additionally, after separation of the intact triglyceride species by reverse-phase HPLC, it was estimated that only 1% of the triglyceride molecules are tri-12:0, whereas the statistically predicted proportion from random acylation at all three *sn* positions would be 7%. Thus, the expression of a lauroyl-CoA preferring plant LPAAT in such C12 producing *Brassica* plants is desirable for enhanced incorporation of 12:0 fatty acyl groups into the *sn*-2 position.

The coconut medium-chain preferring LPAAT may thus be used for enhancing the incorporation of laurate into storage oil in rapeseed. In addition, production of TAG containing other medium-chain fatty acyl groups in *Brassica* and other oilseed crop plants is also desired. (See, for example, WO 92/20236, WO 94/10288 and co-pending application USSN 08/383,756 filed February 2, 1995). As the coconut LPAAT has significant ability to utilize other medium chain lengths, particularly C10 and C14, it also has the potential to enhance the incorporation of these fatty acids into plant TAG. Furthermore, TAGs having shorter chain fatty acyl groups in all three *sn* positions are desirable for various medical applciations. Such TAG

molecules may be obtained by expression of appropriate acyl-ACP thioesterase and LPAAT genes in oilseed crop plants.

Likewise, the expression of any LPAAT which is capable of transferring a medium-chain fatty acyl group into the *sn*-2 position of an LPA substrate is also desired for applications in crop species engineered to contain medium-chain fatty acids. Preferential activity is not required, so long as the capability of medium-chain utilization is present.

Further plant genetic engineering applications for LPAAT proteins of this invention include their use in preparation of structured plant lipids which contain TAG molecules having desirable fatty acyl groups incorporated into particular positions on the TAG molecules. For example, in *Brassica* plants, the *sn*-2 position of TAG contains mainly unsaturated fatty acyl groups. In certain applications, it may be desirable to have saturated fatty acids at the *sn*-2 position, and thus an LPAAT from a different plant source may be identified as having activity on, for example 16:0 or 18:0 acyl-CoA substrates, and used for transformation of *Brassica*.

In addition, in *Brassica* plants which contain high levels of erucic acid (22:1) in their seed oils (high erucic acid rapeseed or HEAR), little or no 22:1 is found in the *sn*-2 position of the TAG molecules. A "tri-erucic" HEAR plant having 22:1 in all three of the TAG *sn* positions is desirable. Such a seed oil might be obtained for example by expression of a C22:1 active LPAAT in HEAR plants. A gene encoding such an LPAAT could be obtained from a plant, such as meadowfoam (*Limnanthes alba*), whose seeds accumulate oil containing erucic acid (22:1) in all three *sn* positions.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available. Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally

susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation. In addition, techniques of microinjection; DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector

capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride and Summerfelt (*Plant Mol. Biol.* (1990) *14*:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed Agrobacterium and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1 Assay for LPAAT Activity

A. Assay for LPAAT Activity in Cell-free Homogenates and Membrane Preparations

To assay for LPAAT activity, the sample is incubated with lysophosphatidic acid (LPA) and acyl-coenzyme A (acyl-CoA) substrates in buffered solution. The acyl substituents of the two substrates are chosen to correspond with the specificity of the enzyme being measured. For example, to measure activity of an LPAAT having preference for medium-chain substrates, lauroyl-LPA (lauroyl-lysophosphatidic acid) and lauroyl-CoA may be used, to measure activity of an LPAAT preferring longer-chain acyl groups, oleoyl-LPA and oleoyl-CoA may be used, to measure activity of an LPAAT preferring very-long chain substrate, erucyl-LPA and erucyl-CoA may be used, and so on, depending upon the type of LPAAT to be tested. The acyl group of one substrate is radioactively labeled in order to detect the product formed. In the examples which follow the acyl substituent of the acyl-CoA substrate is radiolabeled with ^{14}C in the carboxyl group. LPAAT activity results in transfer of this acyl group from the acyl-CoA "donor" substrate to the LPA "acceptor" substrate, converting the latter into the product, phosphatidic acid (PA). LPAAT activity is measured as the amount of radioactive product formed in a given assay time. The PA product is radioactive as a result of the transferred radiolabeled acyl group at the central carbon atom of the molecule, and the quantity of PA formed may be determined by measuring radioactivity of the PA fraction. For this measurement, the PA is first separated from the acyl-CoA substrate by solvent partitioning, or by thin-layer chromatography (TLC).

Acyl[1-^{14}C]-CoA substrates can be purchased from commercial suppliers, such as Amersham (Arlington Heights, IL). Acyl[1-^{14}C]-CoA substrates which cannot be purchased from commercial suppliers (*e.g.* lauroyl[1-^{14}C]-CoA or erucyl[1-^{14}C]-CoA) may be synthesized enzymatically using

the method of Taylor *et al.* (*Analyt. Biochem.* (1990) *184*:311-316). The [1-^{14}C]fatty acids used in the synthesis typically have specific radioactivities of 20 Ci/mol. The radiolabeled acyl-CoA substrate is diluted before use to 12.5μM and stored in 3mM sodium acetate (pH 4.8). Oleoyl-LPA is obtained from commercial suppliers, lauroyl-LPA or erucyl-LPA substrate may be enzymatically synthesized using the method of Ichihara *et al.* (*Eur. J. Biochem.* (1987) *167*:339-3457), or Cao *et al* (*Plant Phys.* (1990) *94*:1199-1206) based on the use of phospholipase D to cleave choline from commercially available lauroyl-lysophosphatidylcholine.

20μl of the sample to be assayed for LPAAT activity is mixed with 217.5μl of an assay ingredient mixture in a 4-ml, screw-cap vial. The components of this mixture are adjusted such that after substrate addition as described below, the final 250μl assay system will contain: 100mM HEPES-NaOH (pH 7.5) (HEPES = N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid], 200mM NaCl, 4% glycerol (v/v), 10mM EDTA (ethylenediaminetetra-acetate, disodium salt), 5mM ß-ME (ß-mercaptoethanol). The LPA substrate is then added (2.5μl) to provide a final concentration of 20μM. Control samples to determine nonenzymatic background "activity" can be prepared by omitting the LPAAT sample or the LPA. The assay incubation is started by addition of 10μl of the 12.5mM radiolabeled acyl-CoA solution so that the final concentration is 5μM. If acyl-CoA concentrations vary slightly from 12.5mM the 10μl volume is changed accordingly to achieve 5μM final concentration, and the volume change accommodated by adjusting the water content of the assay mixture so that the total volume and all concentrations remain unchanged. The incubation takes place in a water bath at 30°C, for 20-30 minutes.

To stop the assay, 0.25ml of 1M KCl in 0.2M H_3PO_4 is added to the vial. At this point, 40μl BSA (bovine serum albumin, fraction V) at 1 mg/ml are added, followed by 0.75ml of a solution of 67 μg/ml unlabeled PA (acting as a "carrier" to facilitate partitioning) in chloroform/methanol (2:1, v/v). The chain lengths of the

PA acyl groups are chosen to correspond to those used in the assay substrates. Upon thorough mixing of these components the radiolabeled PA product of the LPAAT reaction partitions into the organic phase and away from the unreacted acyl-CoA and LPA. The vial is centrifuged briefly at low speed to facilitate the separation of organic (lower) and aqueous (upper) phases. The aqueous phase is then removed and discarded. The total radioactivity extracted into the organic phase is determined by liquid scintillation counting; a 100µl sample of the organic phase is transferred to a 20ml scintillation vial and allowed to dry, and scintillation fluid (3-5ml) is added to the vial. The radioactivity of the sample, after subtraction of the "minus-enzyme" or "minus-LPA" radioactivities, is taken as an approximate indication of the amount of PA formed in the LPAAT-catalyzed reaction and therefore of LPAAT activity.

The determination is an approximation due to the presence of non-PA radioactivity in the organic extract. The non-PA radioactivity results from the partitioning of a small amount of the radiolabeled acyl-CoA substrate into the organic layer along with certain impurities in the acyl-CoA (deriving from impurities in the original radioactive fatty acid used in its preparation), and any free fatty acid resulting from acyl-CoA hydrolysis that may take place.

A more accurate estimation of the LPAAT activity may be obtained by separating the PA product from these contaminants by TLC. The remaining organic phase is applied to a silica TLC plate. Ascending chromatography is carried out for 50 minutes, using the solvent mixture chloroform/pyridine/88% formic acid (50:30:7, v/v). After the plate has dried, the distribution of radioactivity is visualized and quantitated using an AMBIS radioanalytic imaging system (AMBIS Systems Inc., San Diego, California). From prior application of standard lipid components the Rf of the PA is known. The radioactivity associated with the PA spot is expressed as a percentage of the total radioactivity of the assay sample loaded on the plate.

This ratio provides an indication of the proportion of the scintillation counts which represent the PA product, and may be used to correct the counts to obtain the total PA radioactivity formed in the assay.

For a given LPAAT enzyme source, the effects of incubation time and sample concentration on LPAAT activity are determined to define the conditions under which the assay results (PA radioactivity) provide a linear measure of LPAAT activity. Subsequent assays are then conducted within the determined limits.

B. Assay for LPAAT Activity Following Solubilization

After solubilization of LPAAT protein from plant membranes as described below, modification of the above assay conditions is required in order to detect maximum LPAAT activity. This is especially important after the solubilized LPAAT has been chromatographed on at least one column. The important modification to the assay is the addition, at the start of the assay procedure, of 1µl of a concentrated phospholipid (PL) solution to 20µl of the LPAAT-containing sample in a glass vial. The high concentrations of CHAPS (at least 1% w/v) and NaCl (typically 0.5M or greater) in the solubilized LPAAT preparation aid in dispersal of the phospholipids. The phospholipid solution is obtained by sonicating crude soybean phospholipids (L-phosphatidylcholine from soybean, "Type IVs" obtained from Sigma Chemical Company, St. Louis) at 50mg/ml in 0.5% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate) until a uniform suspension is obtained. Synthetic phospholipids (phosphatidyl choline, inositol, or ethanolamine alone or in combination), and turkey egg yolk phospholipid preparation, do not offer significant improvement over the crude soybean material.

The remaining assay ingredients (as described above), with the exception of the acyl-CoA substrate, are then added as 219µl of a mixture. By this addition, the CHAPS and NaCl are diluted to levels which do not hinder enzyme activity, but the solution does not turn cloudy, which suggests that the phospholipids remain dispersed.

Radiolabeled acyl-CoA (10µl, or an appropriately adjusted volume as indicated above) is added to start the LPAAT-catalyzed reaction and the rest of the assay procedure is completed as described above.

The effect of the timing of addition of phospholipids in the assay described above is illustrated in Table 1 below:

TABLE 1

Stage of PL addition	LPAAT Activity (cpm)
At start of assay (control)	914
None added	0
At start of incubation	231
At end of incubation	0

These results demonstrate that the stimulatory action of the phospholipids is greatest when they are added to the LPAAT preparation at the start of the assay procedure, prior to dilution of the CHAPS and NaCl concentrations by addition of the other assay ingredients. Addition of phospholipids after this dilution, or just prior to the addition of partitioning mixture (chloroform/methanol etc.), is less effective or ineffective.

To determine whether this sequence of phospholipid addition is more important for the LPAAT enzyme or for the phospholipids, a second experiment is conducted in which a purified LPAAT preparation (S3 preparation that has been purified sequentially on red 120 agarose and hydroxyapatite columns, Example 5 below) is added just prior to the start of the incubation. In this experiment, the phospholipids are first mixed with Solubilization Buffer and subsequently diluted with the assay components prior to addition of LPAAT activity.

The results demonstrate that the activity obtained by adding the LPAAT preparation just prior to incubation is identical to that obtained when the phospholipids are added at the start of the assay. It is therefore the treatment of the phospholipids, in exposing them to high CHAPS and

NaCl concentrations and then diluting the mixture, that is critical in order to obtain their activation of LPAAT. The final LPAAT activity depends on the phospholipid concentration used, increasing up to 20 µg phospholipid/assay and remaining unchanged from 20 to 50 µg phospholipid/assay. This dependence on phospholipid concentration is independent of S3 concentration. These observations are summarized in Figure 1.

In the following examples, where solubilized and column-chromatographed coconut LPAAT preparations are implicated, the assay data refer to this modified assay method involving the use of soybean phospholipids.

It is not possible to activate the solubilized bay long-chain LPAAT in this way to obtain maximal activity; when the phospholipids are included in the bay assay an alternative reaction occurs, diverting the radiolabeled acyl group from the 18:1-CoA to another product distinguishable from the LPAAT product (PA) by TLC.

Example 2 Preparation of Cell-free Homogenates and Membrane Fractions with LPAAT Activity

A. Coconut LPAAT

Coconuts (*Cocos nucifera*) are obtained from local supermarket stores. For maximum yield of LPAAT activity, immature coconuts referred to as "green", which have a very pale brown or white endocarp (exterior "shell") are used. The endocarp of the coconut is pierced and the "milk" liquid within the hollow interior drained and discarded. The coconut is then broken into fragments so that the white endosperm tissue lining the inside of the endocarp can be dissected and collected. The brown testa between the endosperm and the endocarp is removed and discarded, and the endosperm is frozen by immersion in liquid nitrogen and stored at -70°C for future use. In a typical preparation as described below, 24g of tissue are processed. As individual coconuts may vary considerably with respect to the maturity of the endosperm and therefore the yield of obtainable LPAAT, the endosperm may be sampled to assess the LPAAT content prior to beginning a 24g-scale

preparation. Such a sampling may be accomplished by cutting a hole in the endocarp, approximately 1 inch in diameter. The resulting disc of endosperm is dissected away from the testa and endocarp and processed as described below except that 16ml Extraction Buffer are used for analysis of a 2g powdered endosperm sample.

Frozen coconut endosperm tissue is powdered by impact crushing in a steel mortar and pestle in liquid nitrogen. The powder from 24g of tissue is added to 144ml Extraction Buffer at 0-4°C, and the mixture is blended with a Polytron tissue homogenizer to make a cell-free homogenate. Extraction Buffer contains 50mM HEPES-NaOH (pH 7.5), 3M NaCl, 10mM EDTA, 10mM DIECA (diethyldithiocarbamic acid, sodium salt), 100µM Pefabloc (protease inhibitor available from Sigma Chemical Co. or Boehringer Mannheim), 1µM leupeptin, 0.1µM pepstatin A, 5mM ß-ME. All subsequent steps are performed at 4°C.

The homogenate is filtered through 4 layers of cheesecloth which has been wetted with Extraction Buffer. The remaining solids are enfolded in the cheesecloth and the cheesecloth wrung to extract more liquid. The cheesecloth is then unfolded, the solids wetted with 48ml of Extraction Buffer, and the cheesecloth wrung again. The resulting filtrate is centrifuged at 12,000 x *g* for 30 minutes. The resulting sample contains a floating fat pad and a pellet, which are both discarded, and a supernatant fraction (S1). The supernatant fraction is filtered to remove residual solids using Miracloth (Calbiochem; La Jolla, CA) which has been wetted with Extraction Buffer. This S1 fraction is then dialyzed overnight against 4 liters of Dialysis Buffer (50mM HEPES-NaOH pH 7.5, 1M NaCl, 5mM ß-ME), with one change of buffer. Dialysis membrane having a molecular weight cutoff of 12,000-14,000 is used. The dialyzed S1 material (DS1) is then centrifuged at 12,000 x *g* for 30 minutes and the supernatant fraction again filtered through buffer-wetted Miracloth.

The DS1 supernatant is then centrifuged at 100,000 x *g* for 2 hours. The resulting sample contains a pelleted fraction containing subcellular membranes (P2), and a

supernatant fraction which is discarded. Residual supernatant fraction is removed from the P2 fraction by draining the centrifuge tubes and wiping with paper tissues.

P2 Buffer (100mM HEPES-NaOH (pH 7.5), 200mM NaCl, 20% glycerol (w/v), 10mM EDTA, 5mM ß-ME) is added to the P2 pellets so that when the mixture is transferred to a ground glass homogenizer and homogenized, the total volume of the homogenate will be 2.5ml. The P2 homogenate is divided into aliquots, frozen in liquid nitrogen, and stored at -70°C for future use.

B. California bay LPAAT

A P2 membrane homogenate from immature cotyledons of developing California bay (*Umbellularia californica*) seeds is prepared essentially as described above, except as noted below. The seeds are dissected, and the pale green cotyledons are removed, frozen in liquid nitrogen and stored at -70°C. The frozen bay tissue is powdered in liquid nitrogen as described above. Typically 20g of powdered embryo tissue are homogenized with Modified Extraction Buffer (100mM HEPES-NaOH pH 7.5, 3M NaCl, 10mM DIECA, 100µM PMSF (phenylmethylsulfonyl fluoride), 1µM leupeptin, 0.1µM pepstatin A) in a final volume of 200ml. The homogenate is centrifuged at 10,000 x *g* for 15 minutes, yielding a floating fat pad and a pellet, which are both discarded, and a supernatant fraction (S1).

The S1 fraction is centrifuged at 100,000 x *g* for 90 minutes, yielding a supernatant fraction and a pellet (P2). The P2 pellet, which contains subcellular membranes, is resuspended in approximately 30ml of Modified Extraction Buffer, and centrifuged again at 100,000 x *g* for 90 minutes. The resulting pellet (P3) is resuspended in approximately 2ml Modified P2 Buffer (100mM HEPES-NaOH (pH 7.5), 200mM NaCl, 5% glycerol (w/v), 10mM EDTA). The suspension is then divided into aliquots, frozen in liquid nitrogen and stored at -70°C for future use.

C. Rapeseed LPAAT

A P2 membrane homogenate from immature embryos of developing rapeseed (*Brassica napus*) seeds is prepared

essentially as described above, except as noted below. Immature *Brassica* seeds are harvested from plants grown in growth chambers and greenhouses. The embryos are dissected from the immature seeds and frozen in liquid nitrogen. Approximately 1.66g of *Brassica* embryos are ground in 8ml Modified Extraction Buffer using a chilled mortar and pestle. Since little starting tissue is used, the homogenate is not filtered through cheesecloth, but is centrifuged at 10,000 x *g* for 50 minutes. The supernatant fraction (S1) is then centrifuged at 100,000 x *g* for 2 hours, and the resulting membrane-containing P2 pellet is resuspended in 0.25ml Modified P2 Buffer, frozen in liquid nitrogen, and stored at -70°C for future use.

Example 3 Characterization of LPAAT Activity in Cell-free Homogenates and P2 Membrane Preparations

A. Enzyme activity

Coconut, bay, and rapeseed cell-free homogenates and P2 membrane preparations all display LPAAT activity as measured by the assay described in Example 1A. LPAAT activity is dependent on assay incubation time and varies with the concentrations of substrates and P2 preparation, as expected for enzyme catalysis. Confirmation of the identity of the reaction product as PA can be obtained by incubating the product with phospholipase A2 (available commercially, *e.g.* purified from *Crotalus atrox* venom). Radioactivity is converted to a form which migrates on TLC as free fatty acid. As phospholipase A2 removes the fatty acyl group at the *sn*-2 hydroxyl substituent of PA, this result is consistent with the radioactive LPAAT product being PA radiolabeled at the *sn*-2 position.

B. Substrate specificity

The LPAAT activity involved in triacylglycerol (seed oil) biosynthesis is associated with the cytoplasmic endoplasmic reticulum membranes (sometimes referred to as "microsomes") and prefers acyl-CoAs over acyl-ACPs as donor substrates. A functionally analogous enzyme which is able to utilize both acyl-ACP and acyl-CoA substrates is present

in plant plastids (Harwood, in Crit. Rev. Plant Sci. (1989), vol. 8, pp. 1-43). The coconut P2 preparation will not utilize 12:0-ACP as the LPAAT donor substrate instead of 12:0-CoA. This indicates that the coconut P2 preparation contains the cytoplasmic type of LPAAT appropriate to seed oil biosynthesis. The same assay shows that the 12:0-ACP is not hydrolyzed by the P2 preparation, which demonstrates that the lack of 12:0-ACP utilization by coconut LPAAT is not a result of depletion of 12:0-ACP by hydrolysis. Similarly, the bay P2 preparation will not significantly utilize 18:1-ACP as the LPAAT donor substrate instead of 18:1-CoA. Thus, the bay P2 preparation also contains the endoplasmic reticulum type of LPAAT appropriate to seed oil biosynthesis.

Lysophosphatidylcholine (LPC) acyltransferase (LPCAT) is an enzyme analogous to LPAAT, involved in the biosynthesis of membrane lipids (phosphatidylcholine and derivatives thereof) instead of storage oil. The possibility that the activity measured in the LPAAT assay is not true LPAAT, but rather an inefficient action of LPCAT on the LPAAT substrates, can be tested by direct assay for LPCAT. For example, the LPAAT activity of the coconut P2 preparation with the substrate combination 12:0-CoA + 12:0-LPA is readily measurable, whereas the LPCAT activity of the same preparation with the substrates 12:0-CoA + 12:0-LPC is undetectable. This indicates that the measured medium-chain LPAAT activity is due to an LPAAT enzyme, and not due to an inefficient side-reaction of LPCAT. When the substrates all have 18:1 acyl groups the activities in the LPAAT and LPCAT assays (P2 preparations) are of comparable magnitude. The activities on long-chain substrates may represent either a single acyltransferase enzyme able to use LPA and LPC acceptor substrates, or discrete "long-chain" LPAAT and LPCAT enzymes which are present together.

C. Chain-length Specificity

The LPAAT activities of the P2 membrane preparations are further characterized with respect to chain-length preference for the donor and acceptor substrates. Table 2

below presents results of LPAAT activity analysis of P2 membrane preparations from coconut, bay, and rapeseed. LPAAT activity is measured using a variety of acyl-CoA donor substrates, with the acceptor substrate held constant as 12:0-LPA.

TABLE 2

LPAAT Activity of P2 Membrane Prepartions

Donor (Acyl-CoA) Substrate	LPAAT Activity* from: Coconut	Bay	Rapeseed
6:0	3	1	0
8:0	6	13	2
10:0	43	10	12
12:0	238	14	79
14:0	61	5	16
16:0	21	6	27
18:0	13	6	21
18:1	9	5	218

(* pmol PA formed/30 min assay)

The coconut LPAAT activity demonstrates a dramatic preference for 12:0-containing donor substrate, and also readily utilizes additional medium-chain donor acyl-CoA substrates (10:0- and 14:0-containing acyl-CoA substrates). The bay LPAAT activity when 12:0-LPA is the acceptor substrate demonstrates a preference for medium-chain acyl-CoA substrates (8:0-, 10:0- and 12:0-containing). Rapeseed LPAAT prefers the 18:1 donor when 12:0-LPA is the acceptor, in agreement with previous characterizations.

Similar acyl-CoA preferences are observed when assaying coconut LPAAT activity with 18:1-LPA as the acceptor substrate. However, due to differences in substrate kinetics for 12:0-LPA and 18:1-LPA, direct comparisons of LPAAT activity on different acceptor substrates using a single acyl-CoA donor substrate are difficult to make.

In the examples which follow, "medium-chain" LPAAT refers to activity assayed with 12:0-CoA and 12:0-LPA substrates, and "long-chain" LPAAT refers to activity assayed with 18:1-CoA and 18:1-LPA substrates.

D. Other Properties

Using the bay P2 membrane preparation, many detergents are found to be inhibitory when included in the assay. For example, a long-chain LPAAT activity (18:1-CoA and 18:1-LPA as substrates) in bay P2 preparations is inhibited completely by 0.1% (all concentrations quoted as w/v) octyl glucoside, 0.002% SDS (sodium dodecyl sulfate), 0.005% Zwittergent 3-14 (Calbiochem), 1% Tween 20 or Brij 35, 0.03% Triton X100, and by 0.1% sodium deoxycholate. Exposure of the P2 preparation to higher concentrations than these is possible without permanent loss of enzyme activity, provided the enzyme-plus-detergent mixture is diluted prior to assay to reduce the detergent concentration to a level which is tolerated. For example, the bay P2 preparation can be subjected to a 1-hour exposure to 1.25% Brij 35, 0.5% octyl glucoside, 0.1% Triton X-100, or 2.5% Tween 20 without complete loss of activity, provided the preparation is diluted prior to assay to reduce these detergent concentrations (to 0.025, 0.01, 0.002, and 0.05% respectively).

The detergent CHAPS, used for solubilization as described in the examples which follow, is inhibitory in the coconut medium-chain LPAAT assay at concentrations above 0.1% (w/v). Accordingly CHAPS-solubilized LPAAT must be assayed after dilution to reduce the CHAPS concentration to 0.1% or less. Prior exposure of the coconut P2 preparation to higher CHAPS concentrations, such as 0.5% (w/v), is possible with only partial LPAAT activity loss (50% in this example), provided the dilution is undertaken prior to assay. This phenomenon of tolerance of higher detergent concentrations than can be accepted in the assay provides a basis for screening for solubilization conditions.

The coconut, P2, medium-chain LPAAT activity is unaffected by 0.1mM CoA, 2mM adenosine-5'-triphosphate, or 60µM lysophosphatidylcholine in the assay system.

The long-chain LPAAT activity of the bay P2 preparation varies with pH in the assay, being detectable between pH 6 and 10, high between pH 7 and 9, and maximal at pH 8. The medium-chain LPAAT activity of the coconut P2 preparation also shows little change when the assay is ranged between pH 6.5 and 8.5 (in 0.5 pH increments), and there is a slight preference for pH 8.0.

Example 4 Solubilization of LPAAT Activity

A. Coconut Medium-chain and Bay Long-chain LPAATs

All steps are carried out at 0-4°C. The frozen coconut P2 preparation is thawed and diluted in a volume of P2 Buffer to achieve a protein concentration of 0.94mg/ml P2 protein. Protein concentration is determined by Coomassie dye staining relative to a bovine serum albumin standard. The P2 membrane suspension is then diluted with an equal volume of Solubilization Buffer (50mM HEPES-NaOH, pH7.5, 1.8M NaCl, 20% (w/v) glycerol, 4.5% (w/v) CHAPS, 100µM Pefabloc, 1µM leupeptin, 1 µM Pepstatin A, and 5mM ß-ME), resulting in final concentrations of 1M NaCl, 2.25% (w/v) detergent, and 0.47 mg/ml protein. These component concentrations, and the resulting detergent/protein ratio of 48/1 (w/w), are important for optimal solubilization. The preparation is then incubated on ice for 30 minutes with occasional, gentle stirring, followed by centrifugation at 252,000 x *g* for 2 hours. The resulting supernatant fraction (S3) is filtered through buffer-wetted Miracloth, and may then be stored frozen (-70) with only slight loss of activity. Optimally, it is applied to chromatography columns without an intervening freeze-thaw cycle.

The bay long-chain LPAAT activity in the bay P2 membrane sample is solubilized in the same manner, with the Solubilization Buffer CHAPS and NaCl concentrations being

4% (w/v) and 1M respectively, and the detergent/protein ratio being 58/1 (w/w).

The detergent BIGCHAP (N,N-bis[3-D-gluconamidopropyl]-cholamide) may also be substituted for CHAPS in solubilization of either bay or coconut LPAAT, provided the BIGCHAP concentration in the final mixture is 4% (w/v) and a larger portion of the P2 preparation is used so that the detergent/protein ratio is unchanged.

B. Evidence for Solubilization

"Solubilization" refers to extraction of the LPAAT enzyme from the membranes present in the P2 preparation, in such a way that it then behaves in a manner typical of enzymes that are not membrane-associated. In testing for solubilization of LPAAT activity, the following indications of solubilization are considered:

1) LPAAT activity is not sedimented by high-speed centrifugation equivalent to, or of larger, *g* force than that used to sediment the P2 membranes.
2) LPAAT activity migrates on a size-exclusion chromatography column as though it had a native molecular weight typical of enzymes which are not membrane-associated.
3) Proteins present in the LPAAT preparation will be at least partially separable from each other by column chromatography.

Preparation of the coconut and bay S3 sample having LPAAT activity involves centrifugation at much greater *g* force (252,000 x*g*) than was used to prepare the original P2 material (100,000 x*g*). A substantial proportion (up to 79%) of the LPAAT activity is found in the resulting supernatant fraction (S3 preparation), thereby satisfying the first indication of solubilization.

Figures 2-4 show size-exclusion chromatography of the bay long-chain LPAAT activity, using on-column conditions appropriate to the composition of the LPAAT preparation being applied. As shown in the first graph (Fig. 2), the LPAAT activity of the bay P2 preparation passes through a

Sephacryl S400 size-exclusion column in the manner of a solute having extremely high molecular weight. The use of high-molecular-weight dye to calibrate the column (peak fraction indicated by dotted line labeled "Blue dextran") indicates that the P2 LPAAT activity migrates without penetration into the porous beads of the column, *i.e.* in the "excluded" or "void" volume. This is typical of enzyme activities associated with membrane fragments. The second graph (Fig. 3) shows the Sephacryl S400 behavior of bay long-chain LPAAT which is prepared from P2 material according to the "solubilization" procedure for pea shoot LPAAT, published by Hares and Frentzen (*Planta* (1991) *185*:124-131). This procedure solubilizes the bay embryo LPAAT according to the first indication based on centrifugation. However, it does not lead to significant LPAAT activity which chromatographs as a protein of low molecular weight on a size-exclusion column. Most of the activity continues to elute from the column with very high molecular weight characteristic of membrane fragments. This observation serves to illustrate that the centrifugation criterion alone is insufficient evidence for solubilization.

In contrast, the LPAAT activity of the bay S3 preparation migrates more slowly through a size-exclusion column and emerges after a larger volume of buffer has passed through, as shown in Fig. 4. (In the example shown a Superose 6 column is used, to enable finer resolution of proteins in the 12-200kDa range). This behavior is typical of enzymes whose protein molecules are in free solution, not associated with membrane fragments. From the elution volumes of various enzymes used for test purposes (indicated by dotted lines on the graph) it is possible to calibrate the column, and to conclude that the LPAAT activity of the S3 preparation behaves as though it is a globular protein with an approximate molecular weight of 80kDa. Since most enzymes which are not associated with membranes possess molecular weights in the range 20-100kDa, this "apparent molecular weight" is consistent with the conclusion that the LPAAT has been solubilized. Closely

similar results are obtained with the coconut S3 preparation (assaying medium-chain activity), except that the apparent molecular weight is estimated as 44-50kDa.

Examination of the protein composition of effluent fractions from such size-exclusion chromatography of the coconut preparation, by SDS-PAGE (polyacrylamide gel electrophoresis), shows that many proteins are present. But the composition varies as fractions are examined from one end of the LPAAT activity peak to the other. Such protein fractionation would not be possible if the P2 membranes had not been dispersed into their individual lipid and protein constituents, *i.e.* solubilized. Additional evidence of protein resolution is obtained from application of other types of chromatography to the S3 preparation, as in the examples which follow in the section on purification. Furthermore, by means of additional chromatography it is possible to recognize individual proteins as candidate proteins for the LPAAT enzyme. This observation provides evidence that the LPAAT protein itself is amongst those which are dissociated from the membrane in the solubilization procedure.

C. Properties of Solubilized Coconut LPAAT

Varying the CHAPS and NaCl concentrations, and the detergent/protein ratio (D/P, w/w), of the solubilizaton procedure results in varying degrees of conversion of coconut medium-chain LPAAT activity from the P2 preparation to the S3 preparation (*i.e.* on solubilization as defined by the centrifugation criterion). Figure 5 summarizes the effects of CHAPS concentration (at 1M NaCl) and detergent/protein ratio (D/P, w/w). Lowering the solubilization NaCl concentration below 1M reduces the formation of S3 LPAAT activity (data not shown in figure). The routine solubilization conditions are chosen by selecting the minimum CHAPS concentration for maximal effect (2.25% w/v), and the most effective D/P ratio (48/1 w/w).

Re-examination of the substrate specificity shows that after solubilization and phospholipid-activation coconut LPAAT (S3 preparation) has the same preference for

medium-chain acyl-CoAs as the original P2 activity. Also preserved is the comparable use of 12:0-LPA and 18:1-LPA as acceptor substrates. Assay of the coconut medium-chain LPAAT activity after solubilization (S3 preparation) and reactivation with PLs, using different acyl-CoA substrates, provides the following results (Table 3). In all these assays the acceptor substrate is 12:0-LPA.

TABLE 3

Assay of Solubilized Coconut LPAAT

Acyl-CoA	LPAAT Activity*
6:0	1
8:0	16
10:0	162
12:0	205
14:0	84
16:0	18
18:1	30

*Radioactivity (cpm) of PA product resolved on TLC, after 30 min assay.

Comparing these results with the P2 membrane activities, it is seen that the PL-reactivated, solubilized (S3) activity retains the preference for medium-chain acyl-CoAs.

Increasing the EDTA concentration to 10mM does not affect the LPAAT activity of the coconut S3 preparation. The additions of 1mM Mg^{2+}, Mn^{2+}, or Ca^{2+} are also without significant effect, but the activity is reduced by 50% or more if these ions are added at 10mM. Omitting ß-ME from the assay system results in approximately 50% less LPAAT activity, and concentrations above 5mM also reduce activity. Lowering the assay pH from 7.7 to 6.5 results in a loss of approximately 20% of the LPAAT activity. Raising the pH to 8.0 results in a very slight increase of activity which diminishes again as the pH is raised further to 8.5. The optimum pH is therefore 8.0, but 7.5 is used routinely

to minimize nonenzymatic hydrolysis of acyl-CoAs. There is little change in the activity when the assay concentration of NaCl is varied between 100mM and 200mM, but activity declines steeply as the NaCl concentration is raised above 200mM. Activity is insensitive to changes in glycerol concentration in the assay between 5% and 15% (w/v).

Overnight dialysis of the coconut S3 preparation to remove NaCl results in loss of half of the LPAAT activity. The equivalent NaCl removal using a size-exclusion column results in total activity loss. Stability of the coconut S3 preparation during storage at 4°C is considerably improved once it has been activated with phospholipids.

Example 5 Purification of Coconut Medium-Chain LPAAT

Substantial purification of LPAAT activity relative to the total protein content of the coconut S3 preparation can be obtained by sequential chromatography on columns of red 120 agarose and hydroxyapatite, as follows. The following steps are conducted at 0-4°C for optimal recovery of LPAAT activity.

A. Red 120 Agarose Chromatography

The S3 preparation is diluted to reduce the CHAPS concentration to 1.125% (w/v) and the NaCl concentration to 0.5M, all other conditions remaining the same. It is then applied at 0.5ml/min to a 2.5cm (diam.) x 2cm column of red 120 agarose (Sigma Chemical Co., St. Louis) pre-equilibrated in running buffer containing 50mM HEPES-NaOH, pH 7.5, 20% (w/v) glycerol, 1% (w/v) CHAPS, 0.5M NaCl, 5mM ß-ME. Fractions of 3ml volume are collected. As shown in Figure 6, LPAAT activity is retained by the column while considerable non-LPAAT protein (assayed by the Coomassie dye method) flows through.

The LPAAT activity is eluted by applying running buffer in which the NaCl concentration is adjusted to 2.5M. A sharp peak of protein accompanies the eluted activity. The LPAAT activity recovery from this procedure is typically close to 100%, and typically 85% of the proteins in the coconut LPAAT S3 preparation are removed.

B. Hydroxylapatite Chromatography

The LPAAT-active fractions from the red column, in the buffer containing 2.5M NaCl, are pooled and applied to a 1.5cm (diam.) x 5.7cm HA (hydroxylapatite) column pre-equilibrated with running buffer containing 50mM HEPES-NaOH, pH 7.5, 20% (w/v) glycerol, 1% (w/v) CHAPS, 1M NaCl, 5mM ß-ME. The flow rate is again 0.5ml/min and fractions of 2ml volume are collected. Essentially all of the protein and the LPAAT activity in the sample are bound by the column. The LPAAT activity and bound protein are substantially resolved by elution with a linear, 0-100mM phosphate concentration gradient in the running buffer. These results are illustrated in Figure 7.

The recovery of activity on this column is typically 60-70%. The LPAAT-active fractions are pooled and stored at -70°C after freezing in liquid nitrogen. This active pool forms the starting material for additional purification experiments. Analysis of this preparation by size-exclusion chromatography shows that the LPAAT activity still behaves as though it were a globular protein of apparent molecular weight 44-50kDa. This indicates that the partial purification through the red and HA columns does not result in any significant aggregation of the LPAAT with itself or with other proteins in the preparation, and does not compromise the solubilized state of the LPAAT protein.

In a typical application of this 2-column procedure, the final coconut LPAAT preparation contains 17% of the S1 activity and only 0.4% of the S1 protein. This represents a 40-fold purification of LPAAT relative to the S1 preparation.

Coconut LPAAT activity from the red + HA column sequence still prefers 12:0-CoA over 18:1-CoA as donor substrate, and will still utilize 12:0-LPA and 18:1-LPA as acceptor substrates. It still decreases as the assay NaCl concentration is raised above 200mM, and tolerates freezing and thawing with minimal loss.

Example 6 Identification of Coconut LPAAT Protein

A. SDS PAGE Analysis of LPAAT from Hydroxylapatite Column

The protein composition of the LPAAT preparation obtained from the HA column is analyzed by SDS-PAGE. Visualization of the protein composition of P2, S3, or partially purified S3 preparations by SDS-PAGE requires that the sample not be boiled in the SDS-containing PAGE sample buffer prior to loading the gel. SDS-PAGE analysis reveals the presence of numerous protein species in the enriched LPAAT preparation. Although the protein composition is simplified relative to that of the S1 preparation, additional chromatography is required to identify the protein (or proteins) corresponding to LPAAT activity.

B. LPAAT Chromatography on 12:0-CoA Matrix

Useful resolution of the remaining proteins is obtained by chromatography on a matrix comprising immobilized 12:0-CoA substrate (unlabeled). The column matrix is prepared by attaching the amino group of the CoA moiety of 12:0-CoA to the free carboxyl group of 6-aminohexanoic acid Sepharose 4B. This Sepharose derivative, coupling procedure, and other necessary reagents are obtained from Sigma Chemical Company (St. Louis). A density of coupled 12:0-CoA of 3.9mg/ml wet bead volume can be achieved. A 1 cm-diameter column is prepared with 2 ml of the 12:0-CoA matrix, and equilibrated with running buffer containing 50mM HEPES-NaOH pH 7.5, 20% (w/v) glycerol, 1% (w/v) CHAPS, 0.4M NaCl, 5mM ß-ME at 0.2-0.5 ml/min.

The LPAAT preparation prepared by chromatography from the red and HA columns is diluted with running buffer lacking NaCl, lowering the NaCl concentration to 0.4M, and applied to the 12:0-CoA column. Fractions of 2ml volume are collected. As shown in Figure 8, a small amount of LPAAT activity emerges during the loading stage. However, the majority of the LPAAT activity is bound to the column and can be eluted later by application of a linear 0.4-2M

NaCl gradient in the running buffer. Typically 50-60% of the loaded activity is recovered in this NaCl-eluted peak. If the experiment is repeated with the 6-aminohexanoic acid Sepharose 4B support lacking 12:0-CoA, most of the activity emerges in the loading effluent.

C. SDS PAGE Analysis of LPAAT from 12:0-CoA Column

Analysis of fractions eluted from the 12:0-CoA column by SDS-PAGE and silver-staining shows that considerable resolution of proteins is accomplished. Loading and washing fractions 7 and 10 (Figure 8) contain a complex protein composition comparable to the sample loaded. Salt-eluted fractions 29-36 (Figure 8) contain a much simpler protein composition as shown by two prominent component bands and 6-7 less abundant ones. Several very minor components are also detectable in this sample. The protein composition of such material varies somewhat from one coconut preparation to another, but the considerable purification obtained with the 12:0-CoA column is reproducible. Furthermore, on the SDS-polyacrylamide gel, a band or pair of bands corresponding to proteins having an approximate molecular weight of 27-29kDa (*i.e.* migrating slightly faster in the gel than a marker protein of 31kDa) is most prominent in intensity in fractions 32 and 33. These fractions also contain the maximum LPAAT activity. The 27-29kDa band consistently tracks with LPAAT activity in the various coconut 12:0-CoA column samples examined. This is strong evidence that the 27-29kDa protein (also referred to hereafter as the "29kDa" protein or candidate protein) corresponds to the LPAAT enzyme. The other proteins in fractions 29-36 are most abundant in those fractions which are not at the peak of LPAAT activity, and are therefore less likely to represent LPAAT.

D. Chromatography of Activated LPAAT on 12:0-CoA Matrix

In a modification of the above 12:0-CoA chromatography method, LPAAT is activated by addition of phospholipids prior to loading on the column. In addition, the running buffer is modified to include phospholipids. By these modifications, the LPAAT is maintained in activated form throughout the experiment.

To prepare modified running buffer, 380µl of a detergent solution of phospholipids (50mg/ml in 0.5% (w/v) CHAPS as described for the modified assay) are mixed with 9.5ml of HA column running buffer and this mixture is then diluted by addition of 90ml CHAPS-free buffer comprising 50mM HEPES-NaOH, pH 7.5, 20% (w/v) glycerol, 0.44M NaCl, 5mM ß-ME. This results in final CHAPS and NaCl concentrations of 0.1% (w/v) and 0.5M respectively, and a phospholipid concentration as described for assay of solubilized LPAAT. Enzyme dilution buffer is prepared with phospholipids in the same manner, but such that the final CHAPS and NaCl concentrations are 0.1% (w/v) and 0.46M respectively. This dilution buffer is used to dilute the LPAAT sample from the HA column tenfold prior to loading on the 12:0-CoA column.

When applied in the presence of phospholipids only a small amount of LPAAT activity fails to be retained by the column. The activity may then be eluted at a slow rate as the column is washed with running buffer (Figure 9). Application of 15ml of 0.1mM 12:0-LPA in the running buffer results in the elution of a single large peak of LPAAT activity. Subsequent application of 2.5M NaCl fails to elute additional detectable LPAAT.

Attempts to elute LPAAT from the 12:0-CoA column with 12:0-LPA or 18:1-LPA are unsuccessful (or provide only a very small peak of activity) unless the LPAAT is activated with phospholipids before loading and the column is run with phospholipid-containing buffer in the manner just described. This suggests that LPAAT binds differently to the column when it has been activated with phospholipids, and that this binding is based on recognition of the 12:0-CoA moiety of the column by the catalytic site of the LPAAT protein. The 12:0-LPA elution would then derive from recognition of the 12:0-LPA substrate by the LPAAT catalytic site also. These binding and elution phenomena, if based on the catalytic site, would be expected to be specific for LPAAT and to offer the prospect of considerable purification.

E. SDS PAGE Analysis of LPAAT from Activated 12:0-CoA Column

Examination of the eluted fractions by SDS-PAGE (with silver staining) shows that different proteins are present in the loading effluent, the LPAAT-active fractions, and the 2.5M NaCl effluent. The significantly stained 29kDa LPAAT candidate protein is seen in the LPAAT-active fractions, along with several weakly staining protein bands. The 29kDa protein is not detected in the LPAAT-inactive fractions. These results provide additional evidence that the 29kDa protein represents coconut LPAAT.

F. Additional Chromatographic Analyses

Many other chromatography columns may be tested for their ability to resolve proteins present in active LPAAT preparations from the red + HA column sequence. Columns that are useful in this respect include Pharmacia "Mono Q" anion exchanger, Merck thiophilic agarose, size exclusion columns, and blue 4 agarose. In all these chromatographic analyses, LPAAT activity can be retained by the column and eluted in various ways, always accompanied by a protein or pair of proteins having an apparent molecular weight on SDS-PAGE of approximately 29kDa.

Thus, the chromatographic evidence demonstrates the relationship between LPAAT activity and the protein or proteins migrating with apparent molecular weight of approximately 29kDa on SDS-PAGE. Although this molecular weight does not correspond to the estimate of 44-50kDa for the native enzyme obtained by size-exclusion chromatography, such differences between the molecular weights of denatured proteins on SDS-PAGE and the corresponding proteins in the native state are common. These differences can result from the association of the protein molecules into dimers, tetramers etc. in the native situation, or the binding of limited numbers of detergent molecules etc. during solubilization.

Example 7 Determination of LPAAT Amino Acid Sequence

A. Transfer of LPAAT to Membranes

LPAAT may be further purified for use in determination of amino acid sequence by transfer of the LPAAT preparation resulting from the Red 120 and HA column chromatography purification to nitrocellulose or PVDF membranes following SDS-PAGE. For example, for further use in tryptic digestions, the LPAAT protein is transferred to nitrocellulose. PVDF membranes, such as ProBlott (Applied Biosystems; Foster City, CA) and Immobilon-P (Millipore; Bedford, MA) find preferential use in different methods. For example, transfer to ProBlott is useful for N-terminal sequencing methods. For generation of peptides from cyanogen bromide digestion, Immobilon-P is preferred.

1. *Blotting to Nitrocellulose*: When protein is electroblotted to nitrocellulose, the blotting time is typically 1-5 hours in a buffer such as 25mM Tris (tris(hydroxymethyl)aminomethane), 192mM glycine in 5-20% methanol. Following electroblotting, membranes are stained in 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid for 2 minutes and destained in 2-3 changes of 0.1% (v/v) acetic acid, 2 minutes for each change. These membranes are then stored wet in heat-sealed plastic bags at -20°C. If time permits, blots are not frozen but used immediately for digestion to create peptides for determination of amino acid sequence as described below.

2. *Blotting to PVDF*: When protein is electroblotted to Immobilon P PVDF, the blotting time is generally about 1-2 hours in a buffer such as 25mM Tris/192mM glycine in 20% (v/v) methanol. Following electroblotting to PVDF, membranes are stained in 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid for 5 minutes and destained in 2-3 changes of 50% (v/v) methanol/10% (v/v) acetic acid, 2 minutes for each change. PVDF membranes are then allowed to air dry for 30 minutes and are then stored dry in heat-sealed plastic bags at -20°C. Protein blotted to PVDF membranes such as Pro Blott, may be used directly to determine N-terminal sequence of the intact protein. A

protocol for electroblotting proteins to ProBlott is described below.

B. Protease Digestion and Separation of Peptides

LPAAT protein that has been blotted to nitrocellulose may be subjected to digestion with proteases in order to obtain peptides for sequencing. The method used is that of Aebersold, *et al.* (*PNAS* (1987) *84*:6970).

The LPAAT preparation is transferred to nitrocellulose as described above. The band representing the above-identified 29kDa protein, and also an equal amount of blank nitrocellulose to be used as a control, are cut out of the nitrocellulose membrane. A 1.0ml aliquot of 0.5% polyvinylpyrrolidone (PVP-40, Aldrich, Milwaukee, WI) in 100 mM acetic acid is added to the membrane pieces and the mixture incubated for 30 minutes at 37°C. In order to remove the PVP-40 completely, nitrocellulose pieces are washed with HPLC grade water (6 x 3ml), checking the absorbance of the washes at 214nm on a spectrophotometer. PVP-40 may be more easily removed if bands are not cut into small pieces until after PVP-40 treatment and washing.

Following the PVP-40 treatment, the membrane pieces are minced into small chips (~1mm X 1mm) prior to digestion. The protein is then suspended in trypsin digest buffer (100mM sodium bicarbonate pH 8.2). Acetonitrile is added to the digest mixture to a concentration of 5-10% (v/v). Trypsin is diluted in digest buffer and added to the digest mixture, at a ratio of 1:10 (w/w) protease to protein. Digests are incubated 18-24 hours at 37°C.

Following overnight incubation, the digest reaction is stopped by addition of 10µl of 10% (v/v) trifluoroacetic acid (TFA) or 1µl 100% TFA. The peptides in the digest mixture are separated on a Vydac reverse phase C18 column (2.1mm x 150mm) installed in an Applied Biosystems (Foster City, CA) Model 130 High Performance Liquid Chromatograph (HPLC). Mobile phases used to elute peptides are: Buffer A: 0.1mM sodium phosphate, pH2.2; Buffer B: 70% acetonitrile in 0.1mM sodium phosphate, pH2.2. A 3-step gradient of 10-55% buffer B over two hours, 55-75% buffer B over 5 minutes, and 75% buffer B isocratic for 15 minutes

at a flow rate of 50ml/minute is used. Peptides are detected at 214nm, collected by hand, and stored at -20° C.

Other proteases may also be used to digest the LPAAT protein in appropriate digest buffers, for example, endoproteinase gluC buffer (25mM ammonium carbonate/1mM EDTA, pH 7.8), or endoproteinase Asp-N buffer (0.05M sodium bicarbonate pH 8.0). In addition, buffer conditions, such as temperature may vary, for example endoproteinase gluC digestion is conducted at room temperature. However, the protocols for digestion, peptide separation and purification are substantially as described above for digestion with trypsin.

C. Cyanogen Bromide Cleavage and Separation of Peptides

Cyanogen bromide cleavage may be performed on LPAAT protein using the methodology described in the Probe-Design Peptide Separation System Technical Manual from Promega, Inc. (Madison, WI). The LPAAT protein preparation is blotted to a PVDF membrane as described above. The portion of the membrane containing the transferred 29kD band is cut from the blot, placed in a solution of cyanogen bromide in 70% (v/v) formic acid, and incubated overnight at room temperature. Following this incubation the cyanogen bromide solutions are removed, pooled and dried under a continuous nitrogen stream using a Reacti-Vap Evaporator (Pierce, Rockford, IL), or evaporated using a Speed-Vac. Additional elution of cyanogen bromide peptides from PVDF may be conducted to ensure complete removal, using a peptide elution solvent such as 70% (v/v) isopropanol, 0.2% (v/v) trifluoroacetic acid, 0.1mM lysine, and 0.1mM thioglycolic acid. The elution solvents are then removed and added to the tube containing the dried cyanogen bromide solution, and dried as described above. The elution procedure may be repeated with fresh elution solvent. 50µl of HPLC grade water is then added to the dried peptides and the water removed by evaporation in a Speed-Vac (Savant, Inc., Farmingdale, NY).

Peptides generated by cyanogen bromide cleavage are separated using a Tris/Tricine SDS-PAGE system similar to

that described by Schägger and von Jagow (*Anal. Biochem.* (1987) *166*:368-379). Gels are run at a constant voltage of 125-150 volts for approximately 1.5 hours or until the tracking dye has begun to run off the bottom edge of the gel. Gels may be pre-soaked in transfer buffer (125mM Tris, 50mM glycine, 10% (v/v) methanol) for 15-30 minutes prior to transfer. Gels are blotted to ProBlott sequencing membranes (Applied Biosystems, Foster City, CA) for 2 hours at a constant voltage of 50 volts. The membranes are stained with Coomassie blue (0.1% in 50% (v/v) methanol/10% (v/v) acetic acid) and destained for 3X 2 min. in 50% (v/v) methanol/10% (v/v) acetic acid. Membranes are air-dried for 30-45 minutes before storing dry at -20° C.

Peptides blotted on to ProBlott can be directly loaded to the sequencer cartridge of the protein sequencer without the addition of a Polybrene-coated glass fibre filter. Peptides are sequenced using a slightly modified reaction cycle, BLOT-1, supplied by Applied Biosystems. Also, solution S3 (butyl chloride), is replaced by a 50:50 mix of S1 and S2 (n-heptane and ethyl acetate). These two modifications are used whenever samples blotted to ProBlott are sequenced.

D. N-terminal Sequencing of Proteins and Peptides

Sequencing is performed by Edman degradation on an Applied Biosystems 477A Pulsed-Liquid Phase Protein Sequencer; phenylthiohydantoin (PTH) amino acids produced by the sequencer are analyzed by an on-line Applied Biosystems 120A PTH Analyzer. Data are collected and stored using an Applied BioSystems model 610A data analysis system for the Apple Macintosh and also on to a Digital Microvax using ACCESS*CHROM software from PE NELSON, Inc. (Cupertino, CA). Sequence data are read from a chart recorder, which receives input from the PTH Analyzer, and is confirmed using quantitative data obtained from the model 610A software.

For peptide samples obtained as peaks from an HPLC, the sample is loaded on to a Polybrene coated glass fiber filter (Applied Biosystems, Foster City, CA) which has been pre-washed. For peptides which have been reduced and

alkylated, a portion of the PTH-amino acid product material from each sequencer cycle is counted in a liquid scintillation counter. For protein samples which have been electroblotted to Immobilon-P, the band of interest is cut out and then placed above a Polybrene coated glass fiber filter, pre-washed as above and the reaction cartridge is assembled according to manufacturer's specifications. For protein samples which have been electroblotted to ProBlott, the glass fiber filter is not required.

In order to obtain protein sequences from small amounts of sample (5-30 pmoles), the 477A conversion cycle, the $S4_B$ solvent and the 120A analyzer program are modified as described by Tempst and Riviere (*Anal. Biochem.* (1989) *183*:290).

Amino acid sequence of peptides generated from the 29kDa LPAAT by trypsin digestion as described above are as follows:

SQ1256 (SEQ ID NO:1)	NLSLIIFPEGTr
SQ1262 (SEQ ID NO:2)	YFSPIK
SQ1282 (SEQ ID NO:3)	VRPAPITVK

Amino acid seqeunce of peptides generated from the 29kDa LPAAT by AspN digestion as described above are as follows:

SQ1271 (SEQ ID NO:4)	TGTHLa
SQ1272 (SEQ ID NO:5)	VEMIHaly
SQ1276 (SEQ ID NO:6)	slrvrpapitvk
SQ1281 (SEQ ID NO:7)	FSPIKT

The amino acid sequence is represented using the one letter code. Amino acids represented by lower case letters represent residues which were identified with a lesser degree of confidence.

E. Homology of LPAAT Peptide to Acyltransferase Proteins

The amino acid sequence of the LPAAT tryptic peptide SQ1256 described above is compared to known protein sequences in a computer data bank by computer aided homology search. Significant homology is found between the LPAAT peptide and the LPAAT encoded by the *E. coli plsC*

gene. A six amino acid stretch of the 12 amino acid coconut LPAAT tryptic peptide is an identical match to amino acids 145-150 of the *E. coli* LPAAT (Coleman *et al.*, *supra*). In addition, this same conserved six amino acid sequence is also found at amino acids 154-159 of a yeast acyltransferase protein encoded by the *SLC*1 gene. Additional regions of homology with the *E. coli plsC* and yeast *SLC*1 gene products are found in coconut LPAAT amino acid sequence as determined by translation of nucleic acid sequences of LPAAT PCR sequences described in Example 9.

Example 8 Preparation of cDNA Library

A. Total RNA preparation

This procedure is an adaptation of the DNA isolation protocol of Webb and Knapp (D.M. Webb and S.J. Knapp, (1990) Plant Molec. Reporter, 8, 180-185). The following description assumes the use of 1g fresh weight of coconut tissue. Frozen immature endosperm tissue (from "green" coconuts as described for LPAAT purification) is powdered by grinding under liquid nitrogen. The powder is added to 10ml REC buffer (50mM Tris-HCl, pH 9, 0.8M NaCl, 10mM EDTA, 0.5% w/v CTAB (cetyltrimethyl-ammonium bromide)) along with 0.2g insoluble polyvinylpolypyrrolidone, and ground at room temperature. The homogenate is centrifuged for 5 minutes at 12,000 *xg* to pellet insoluble material. The resulting supernatant fraction is filtered through Miracloth into a 3ml phenol/chloroform preparation (phenol-saturated water/chloroform, 1/1 v/v, set to pH 7 with solid Tris base). After brief centrifugation as above to facilitate phase separation the upper phase is removed and the lower phase discarded. The upper phase is partitioned again with chloroform, and the top phase is again recovered.

The RNA is then precipitated by addition of 1 volume ethanol and collected by brief centrifugation as before. The RNA pellet is redissolved in 1 ml autoclaved 0.05% (w/v) DEPC (diethylpyrocarbonate), and reprecipitated by the addition of 1ml 4M potassium acetate (pH 5), 0.05% (w/v) DEPC and incubation on ice for 2 hours. After

collection by brief centrifugation, the RNA pellet is redissolved in 0.4 ml 0.05% (w/v) DEPC and extracted once more with phenol/chloroform as described above. Sufficient 3M potassium acetate (pH 5), 0.05% (w/v) DEPC is added to make the mixture 0.3M in acetate, followed by addition of two volumes of ethanol to precipitate the RNA. This final RNA precipitate is dissolved in 0.1ml 0.05% (w/v) DEPC and stored frozen.

When a total RNA preparation for meadowfoam, or other plant tissue, is desired, the Webb and Knapp protocol described above is modified as follows. First, frozen developing seed tissue (13-20 days post pollination) from meadowfoam is used. The 10ml REC buffer is the same as described above but with the addition of 0.1% ß-mercaptoethanol. After centrifugation, the resulting supernate fraction is extracted with chloroform.

The RNA is then precipitated by addition of 1 volume RECP buffer (50mM Tris-HCL, pH 9, 10mM EDTA, 0.5% w/v CTAB, 0.1% ß-mercaptoethanol) and collected by brief centrifugation as before. The RNA pellet is redissolved in 1ml 0.4m NaCL, extracted with 0.5ml phenol/chloroform (1:1) and reprecipitated by the addition of 2ml ethanol. After collection by brief centrifugation, the RNA pellet is dissolved in 0.4ml H_2O. Optionally, 100mg of the total RNA can be purified on an RNeasy cellulose column (Qiagen, Inc. Chatsworth, CA.) according to the manufacturer's protocol.

B. Construction of cDNA Library

A cDNA library is constructed using Stratagene's (San Diego, CA) "UniZap" system. When a coconut cDNA library is prepared, the following modifications are useful. Forty µg of total RNA from coconut endosperm are reverse-transcribed in a 50µl reaction volume as follows: The RNA, in H_2O, is heated at 65°C for 20 minutes and chilled on ice. The first-strand synthesis is carried out as recommended by Stratagene, with the substitution of 600U "Superscript" reverse transcriptase, "Superscript" 1st-strand buffer, and DTT, all as supplied by BRL (Bethesda, MD). The reaction mixture is incubated at 60°C for 45 minutes. The remaining steps in the library synthesis are performed as recommended

in the Stratagene "UniZap" protocol. The unamplified cDNA library obtained by this procedure contains 1.4 X 10^6 clones with an average insert size of 1.25 kb.

When a meadowfoam cDNA library is prepared, 40µg of total RNA from meadowfoam endosperm are reverse-transcribed in a 50µl reaction volume where the DNA, in H_2O, is heated at 65° C for 20 minutes and chilled on ice. The first strand synthesis is carried out as recommended by Strategene with the only other modification being that the reaction mixture is incubated at 45° C.

Example 9 Isolation of LPAAT-Encoding Sequences

DNA sequences encoding LPAAT peptides are obtained from an LPAAT-containing plant source of interest using synthetic oligonucleotides designed from LPAAT peptide sequences. The LPAAT nucleic acid sequences may be obtained by amplification of DNA by polymerase chain reaction (PCR) using oligonucleotides as primers, or alternatively, by screening a cDNA or genomic DNA library by radiolabeling the oligonucleotides or previously isolated sequences for use as probes.

A. Synthetic Oligonucleotides

In general, for use as PCR primers from single stranded DNA template reverse-transcribed from mRNA, oligonucleotides containing the sense orientation sequence corresponding to LPAAT peptide encoding sequences are prepared. These oligonucleotides are used as primers for the "forward" amplification reaction to produce sense strand DNA.

For the "reverse" reaction for amplification of the non-coding DNA strand, an oligonucleotide may be designed to be identical to a portion of a primer used to prepare DNA template for PCR. Alternatively, oligonucleotides which contain sequence complementary to LPAAT peptide encoding sequences may be used in combination with a "forward" LPAAT oligonucleotide primer as described above.

Where the LPAAT peptide sequences contain amino acids which may be encoded by a number of different codons, the

forward or reverse primers may be "degenerate" oligonucleotides, i.e. containing a mixture of all or some of the possible encoding sequences for a particular peptide region. To reduce the number of different oligonucleotides present in such a mixture, it is preferable to select peptide regions which have the least number of possible encoding sequences when preparing the synthetic oligonucleotide for PCR primers. Similarly, where the synthetic oligonucleotide is to be used to directly screen a library for LPAAT sequences, lower degeneracy oligonucleotides are preferred.

In addition to LPAAT encoding sequence, oligonucleotides for primers in PCR will contain additional, non-LPAAT sequences to aid in cloning of the PCR products into convenient plasmid vectors. The non-LPAAT sequences may be for restriction digestion sites which may be used to clone the PCR fragments into various plasmids, or may be designed to contain sequences useful for cloning into a particular commercially available vector. For example, the synthetic oligonucleotides described below contain sequences useful for cloning using the CLONEAMP™ system (GIBCO BRL; Gaithersburg, MD), which utilizes UDG (uracil DNA glycosylase) for directional cloning of PCR products (Nisson *et al.* (1991) *PCR Meth. and Appl. 1*:120-123).

Following are sequences of synthetic oligonucleotides which may be used to obtain LPAAT sequences. The oligonucleotide names reflect the particular LPAAT peptide fragment numbers as listed in Example 7D. The letter "F" in the oligonucleotide name designates a PCR forward reaction primer. The letter "R" designates a PCR reverse reaction primer. The letter "P" designates an oligonucleotide to be radiolabeled for use as a probe in cDNA or genomic library screening. The underlined portion of the PCR primers indicates the LPAAT peptide encoding sequence.

SQ1256-1	5'	CUACUACUACUAATHATHTTYCCOGARGG 3'
SQ1256-R1	5'	CAUCAUCAUCAUCCYTCOGGRAAIATIAT 3'
SQ1262-F1	5'	CUACUACUACUATAYTTYWSOCCOATHAA 3'
SQ1262-R1	5'	CAUCAUCAUCAUYTTDATOGGOSWRAARTA 3'
SQ1272-F1	5'	CUACUACUACUAGTOGARATGATHCA 3'
SQ1272-R1	5'	CAUCAUCAUCAURTGDATCATYTCOAC 3'
SQ1272-P1	5'	RTGDATCATYTCOAC 3'
SQ1272-P2	5'	RTGDATCATYTCNAC 3'

An oligonucleotide, TSYN, is used for reverse transcription from poly(A)+ or total RNA to prepare single-stranded DNA for use as a PCR template. In addition to a poly(T) region for binding to the mRNA poly(A) tail, the oligonucleotide contains restriction digestion sequences for *Hind*III, *Pst*I and *Sst*I. The sequence of TSYN is as follows:

TSYN	5'	CCAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTT 3'

An oligonucleotide, 5' RACEAMP, is useful in the reverse reaction of PCR for amplification of the antisense strand of an LPAAT encoding sequence. It is noted that where the template for PCR is single stranded DNA reverse-transcribed from mRNA, the reverse reaction will not occur until completion of the first forward reaction. The first strand reaction results in production of a sense strand template which may then be used in amplification of the antisense DNA strand from the reverse primer. In addition to a region of identity with TSYN (restriction digest region), 5'RACEAMP contains the 5' CAU stretch used in the CLONEAMP™ cloning system. The sequence of 5'RACEAMP is as follows:

5'RACEAMP	5'	CAUCAUCAUCAUAAGCTTCTGCAGGAGCTC 3'

Additional sequences which may be useful to obtain LPAAT sequences are set forth below. These primers were developed in the course of recovering meadowfoam LPAAT

sequence based upon observed sequence homologies between the coconut, *E. Coli* and yeast LPAATs:

F1: CAUCAUCAUCAUGAATTCAAGCTTATHWWBATHKSNAAYCA
F2: CAUCAUCAUCAUGAATTCAAGCTTACNGTNACNRTNGSNAARAA
R3: CUACUACUACUAGGATCCGTCGACYTTYTTNWCNAYNGTNACNGT
F4: CAUCAUCAUCAUGAATTCAAGCTTYCCNGARGGNACNMG
R5: CUACUACUACUAGGATCCGTCGACKNGTNCCYTCNGGRAA

Comparison of the coconut and meadowfoam LPAAT clones shows several regions containing stretches of 6 or more amino acids identical between the two proteins are suitable for design of degenerate oligonucleotides to use to PCR amplify cDNA clones encoding LPAAT from other plant species. Because coconut and meadowfoam are from different classes (monocot vs dicot) of the flowering plants, peptide sequences that are conserved between these species are likely to be conserved amongst all plants. The oligonucleotides that encode these conserved regions will allow PCR amplification of LPAAT encoding DNA sequences in cases where the E. coli, yeast, and coconut homologies failed. In the c-terminal region of the protein already sequenced, the following peptide sequences are suitable for design of degenerate oligonucleotides:

FPEGTRS (amino acids 202-208 of the attached alignment)
GRLLPFKKGF (amino acids 211-220 of the attached alignment)
LTGTHLAWRK (amino acids 236-245 of the attached alignment)
PITVKY (amino acids 254-269 of the attached alignment)

Any 6 or more contiguous amino acids can be used to design oligonucleotides of 17 or more nucleotides. When the protein sequence of the n-terminal portion of the meadowfoam LPAAT is determined, more peptide sequences suitable for degenerate oligonucleotide design will be determined. DNA sequences such as CAUCAUCAUCAUGAATCAAGCTT may be added to the 5′ end of the forward primers and CUACUACUACUAGGATCCGTCGAC may be added to the 5′ end of the reverse primers to facilitate cloning of the PCR products.

The nucleotide base codes for the above oligonucleotides are as follows:

A = adenine T = thymine Y = cytosine or thymine
C = cytosine U = uracil R = adenine or guanine
G = guanine I = inosine O = inosine or cytosine
H = adenine, cytosine or thymine
N = adenine, cytosine, guanine or thymine
W = adenine or thymine
S = guanine or cytosine
B = guanine, cytosine or thymine
K = guanine or thymine
M = adenine or cytosine

B. PCR Reactions

Poly(A)+ RNA is isolated from total RNA prepared from coconut tissue as described in Example 8. Single-stranded cDNA is prepared from poly(A)+ or total RNA by reverse transcription using Superscript reverse transcriptase (BRL) and TSYN as the oligonucleotide primer. The reaction is conducted according to manufacturer's directions, except that the reaction is run at 45°C rather than 37°C. The coconut single-stranded cDNA is used in PCR reactions 1-9 as set forth below.

PCR is conducted in a Perkin Elmer Cetus GeneAmp PCR System 9600 PCR machine using reverse transcribed single-stranded cDNA as template. Commercially available PCR reaction and optimization reagents are used according to manufacturer's specifications. The following reactions using the above described synthetic oligonucleotides are run:

Reaction	Forward Primer	Reverse Primer
1	SQ1256-1	5'RACEAMP
2	SQ1262-F1	5'RACEAMP
3	SQ1272-F1	5'RACEAMP
4	SQ1262-F1	SQ1256-R1
5	SQ1262-F1	SQ1272-R1
6	SQ1256-1	SQ1262-R1
7	SQ1256-1	SQ1272-R1
8	SQ1272-F1	SQ1256-R1
9	SQ1272-F1	SQ1262-R1
10	F1	R3
11	F1	R5
12	F2	F5
13	F4	5' RACEAMP

DNA fragments generated in PCR reactions are cloned into pAMP1 (CLONEAMP™ system; GIBCO BRL). The DNA sequence of the cloned fragments are determined to confirm that the cloned fragments encode LPAAT peptides.

Sequence of two coconut PCR products, 23-2 and 23-4, from reaction 7, and one coconut PCR product, 10-1, from reaction 6, are confirmed as encoding LPAAT peptides by DNA sequence and translated amino acid sequence analysis. The sequences of these reactions are provided in Figures 10-12. Sequences of two other PCR products, MeadLPAAT 15 and MeadLPAAT 20, from reaction 13, are also confirmed as encoding LPAAT peptides by DNA sequence (Figures 14 and 15) and translated amino acid analysis (Figure 16).

In Figure 10, DNA and translated amino acid sequences of clone 23-2, obtained by PCR with oligonucleotides SQ1256-1 and SQ1272-R1, are shown. Translation of the DNA sequence in portions of two different reading frames is required to locate the expected coconut LPAAT peptide regions encoded in the PCR primers. Translated sequence of nucleotides 13-30 corresponds to amino acids 5-10 of the tryptic peptide SQ1256 (SEQ ID NO:1), which were encoded by the forward primer. Nucleotides 245-259 correspond to amino acids 1-5 of the AspN peptide SQ1272 (SEQ ID NO:5),

encoded by the reverse primer. Translation of nucleotides 32-259 corresponds to additional LPAAT peptide sequences. For example, nucleotides 32-37 encode amino acids 11-12 of SQ1256, although in a different translation frame from the sequence encoding amino acids 5-10 of SQ1256. From this information, as well as by comparison to sequence of clone 23-4 (Figure 11), it appears that an additional nucleotide not present in LPAAT encoding sequence was incorporated into the LPAAT encoding sequence (most likely an extra guanine in nucleotides 27-30) during the polymerase chain reaction.

In addition to the expected LPAAT amino acid sequences from the forward and reverse primers, the 23-2 translated sequence corresponds to other LPAAT peptide sequences. Nucleotides 125-142 encode the AspN peptide SQ1271 (SEQ ID NO:4); nucleotides 155-190 encode the AspN peptide SQ1276 (SEQ ID NO:6), as well as tryptic peptide SQ1282 (SEQ ID NO:3) (SQ1282 is identical to amino acids 4-12 of SQ1276); and nucleotides 191-211 encode the AspN peptide SQ1281 (SEQ ID NO:7) and tryptic peptide SQ1262 (SQ ID NO:2).

DNA sequence of a second coconut clone, 23-4, of a larger reaction 7 PCR product is shown in Figure 11. In this sequence, the last two amino acids of the SQ1256 peptide are encoded in frame with amino acids 5-10 (encoded by the PCR primer). The difference in size between the 23-4 insert (approximately 360 bp) and the 23-2 product (approximately 270 bp) is apparently due to the presence of an unprocessed intron in the 23-4 sequence (untranslated sequence at nucleotides 70-157 of Figure 11). The presence of the intron is likely due to an unprocessed LPAAT RNA in the total RNA (as opposed to poly(A)+) used to generate the single-stranded cDNA PCR template.

Excluding the intron and PCR primer regions, the LPAAT sequences of the inserts in 23-2 and 23-4 match at all but a single nucleotide, namely nucleotide 90 of 23-2, which is a thymine, and corresponding nucleotide 177 of 23-4, which is a cytosine. This nucleotide difference also results in a difference in the translated amino acid sequence of 23-2 and 23-4. A leucine is encoded by nucleotides 89-91 in 23-

2, and a proline is encoded by corresponding nucleotides 176-178 of 23-4.

DNA sequence of the approximately 220 bp insert in the cloned PCR product of reaction 6, 10-1, is provided in Figure 12. The LPAAT encoding sequence of this clone, with the exception of the PCR primer regions, is identical to that of 23-4 in the shared region.

For reactions 10-13, the following procedures were followed:

Meadowfoam, nasturtium, and *Brassica* RNAs were purified on RNeasy columns (Qiagen Inc., Chatsworth, CA). 2.5 µg of the purified RNA was used in 20µl first strand cDNA reactions using Superscript reverse transcriptase (Gibco/BRL, Bethesda, MD) according to the manufacturers protocol. After first strand cDNA synthesis, the volume of the reaction was increased to 40µl by the addition of 20µl of water, and unincorporated nucleotides and small cDNA synthesis products were removed by purifying the product on MicroSpin S-400 spin columns (Pharmacia Inc., Piscataway, NJ). PCR was performed in 50µl reactions containing 1µl of the purified first strand cDNA, several of the primer combinations shown previously, and other standard reaction components as specified by the manufacturer (Perkin Elmer, Foster City, CA). PCR reactions were carried out in a Perkin Elmer PCR thermal cycler (model 9600. The reactions were heated to 96°C for 5 minutes, reduced to 72°C for 5 minutes (during which time the Taq polymerase was added); the reaction temperature was reduced to 50°C over a period of 10 minutes, and raised to 72°C for 5 minutes. This was followed by 35 cycles of: 94°C for 15 seconds, rapid reduction of the temperature to 65°C, slow reduction of the temperature to 50°C with a 3 minute ramp time, and 72°C for 60 seconds. The PCR products were analyzed by agarose gel electrophoresis. Smears were visible in all reactions with bands of discrete sizes visible against the smear. Using primers F4 and 5'RACEamp, the reactions containing Brassica, Nasturtium, and Meadowfoam cDNA had visible bands of approximately 350 nucleotides and 550 nucleotides. This

indicates that the PCR reactions yielded multiple sized PCR products.

In Figures 14 and 15 respectively, DNA and translated amino acid sequences of clone MeadLPAAT 15 and clone MeadLPAAT 20 obtained by PCR with oligonucleotides F4 and 5'RACEAMP are shown. Translated sequence of nucleotides 11-28 correspond to the amino acids which were encoded by the forward primer. Nucleotides 489-517 correspond to the reverse primer of clone MeadLPAAT 15 and of nucleotides 485-508 of clone MeadLPAAT 20. Translation of nucleotides 11-313 corresponds to LPAAT coding sequences.

C. Library Screening

1. *Synthetic oligonucleotide as probe*: Useful hybridization solutions for library screening with oligonucleotide probes, such as SQ1272-P1 or SQ1272-P2, include tetraalkylammonium salt solutions, such as described by Jacobs, *et al.* (*Nucl. Acids Res.* (1988) *16*:4637-4650). Appropriate hybridization conditions, such as hybridization and washing temperatures, may also be determined by Northern analysis of RNA blots containing RNA from the enzyme source, ie. coconut endosperm. The oligonucleotide may then be radiolabeled and hybridized with clones from the coconut cDNA library described above, or from a coconut genomic library, in order to identify clones containing sequences encoding LPAAT peptides.

2. *PCR product as probe*: LPAAT DNA fragments obtained by PCR as described above may also be radiolabeled and used as probes for coconut or other plant LPAAT clones (Maniatis, *supra*). For example, to obtain coconut LPAAT clones, an approximately 280 bp fragment of clone 23-2 containing the LPAAT encoding region is obtained by digestion of 23-2 with *Xba*I and *Sal*I and isolation of the resulting approximately 280 bp fragment. The fragment is radiolabeled by random priming using a random labeling kit (Stratagene; La Jolla, CA). Approximately 240,000 plaques of the coconut endosperm cDNA library in the UniZap phage are plated, lifted onto nylon membrane filters and hybridized to the labeled LPAAT 23-2 fragment. Hybridization is conducted at 42°C in hybridization

solution containing 50% formamide, 5XSSC (1X SSC = 0.15 M NaCl; 0.015 M Na citrate), 0.1% SDS, 0.1 mg/ml salmon sperm DNA, 10X Denhardt's solution. The filters are washed in 1X SSC, 0.1% SDS at room temperature for 30 minutes, followed by two 30 minute washes in the same solution at 37°C. A total of 32 hybridizing plaques are identified. The identified plaques are replated and hybridization with the radiolabeled plaque is repeated to obtain purified cultures of 30 of the LPAAT containing phage. The LPAAT cDNA fragments are excised from the UniZap phage vector according to manufacturer's (Stratagene) directions. Briefly, a helper phage system is used which results in automatic excision and recircularization of excised cDNA to generate subclones in a pBluescript SK- (Stratagene) phagemid vector. The LPAAT subclones are further analyzed to determine the lengths of the various inserts and 3' non-coding sequences are obtained and analyzed to determine the number of classes of LPAAT clones.

Although cDNA clones of various sizes are obtained, DNA sequence analysis of the 3' portions of 26 of the clones indicates that they are from the same gene. The clones vary in sequence length at both the 5' and the 3' ends. The variation at the 3' ends indicates that more than one polyadenylation site is used. DNA sequence and translated amino acid sequence of full length clone COLP4 (pCGN5503) is provided in Figure 13.

The calculated molecular mass of the translated LPAAT protein of COLP4 is approximately 34.8 kD, and the estimated isoelectric focusing point is 9.79. The calculated molecular mass is not inconsistent with the observed 27-29 kD value from SDS-PAGE.

Two additional clones having the same 5' sequence as COLP4 were also examined. Each of these clones contained a deletion in the LPAAT encoding region. In clone COLP25, a 99 bp region (bases 721-819 of Figure 13) is deleted. The proper frame for translation is maintained, resulting in a translated protein lacking a 33 amino acid LPAAT peptide region. In clone COLP10, a 49 bp region (bases 820-868 of

Figure 13) is deleted, and the LPAAT reading frame is not maintained.

To obtain meadowfoam clones, a similar procedure as described above is used.

An approximately 510 bp fragment of clones MeadLPAAT 15 and MeadLPAAT 20 containing the LPAAT encoding region is obtained by digestion of the clones with *EcoRI* and *PstI* and isolation of the resulting approximately 510 bp fragment. The fragment is radiolabeled by random priming using a random labeling kit (Pharmacia, Piscataway, N.J.). Approximately 240,000 plaques of the Meadowfoam endosperm cDNA library in the UniZap phage are plated, lifted onto nylon membrane filters and hybridized to the labeled LPAAT fragment. Hybridization is conducted at 37° C in hybridization solution containing 30% formamide, 5XSSC (1X SSC = 0.15 M NaCl; 0.015 M Na citrate), 0.1% SDS, 0.1 mg/ml salmon sperm DNA, 10X Denhardt's solution. The filters are washed exhaustively in 1X SSC, 0.5% SDS at 55° C. A total of 41 hybridizing plaques are identified. As described above, the identified plaques are replated and hybridization with the radiolabeled plaque is repeated to obtain purified cultures of the LPAAT containing phage. The LPAAT cDNA fragments are excised from the UniZap phage vector according to manufacturer's (Stratagene) directions and further analyzed to determine the lengths of the various inserts. 5' and 3' non-coding sequences are obtained and analyzed to determine the number of classes of LPAAT clones. Sequence of the 5' ends of 14 cDNAs indicated few nucleotide differences. A full length clone, Melp2 was selected for construct preparation. Melp4 was the clone with the most differences from MELP2, and the entire clone was sequenced. It is 43 ntps shorter at the 3' end (has a different polyadenylation site), and is not quite a full length clone. Within the regions that are found in both clones, there are 21 nucleotide differences, resulting in 4 amino acid changes.

DNA sequencing showed that two clones isolated by PCR from meadowfoam cDNA encode a protein with homology to the coconut LPAAT. The clones are approximately 510

nucleotides long and contain DNA sequence that encodes the C-terminal 102 amino acids of the meadowfoam LPAAT. Slight differences in the lengths of the two clones are due to different lengths of the poly A tails that were included in the clones. Differences between the two clones in the first 27 nucleotides of the DNA sequence arise from the degenerate nature of the primers used in the PCR reaction, and do not represent real differences in the sequences of the genes that have been cloned. MEADLPAAT20 also differs from MEADLPAAT15 by the presence of a G in the polyA tail (nucleotide 494 of MEADLPAAT20). This G is most likely an artifact of PCR amplification, because this is part of the 5'RACEAMP primer that is an A. Besides the above mentioned differences between the clones, there are 10 nucleotide differences between the two clones indicating that the sequences are diverged by about 2%. The amino acid sequences encoded by the two clones differ by two amino acids, also indicating that the proteins differ by about 2%. Comparison of the coconut clone and meadowfoam LPAAT PCR clones shows that 71/102 amino acids are identical between meadowfoam and coconut (70% identity, see the attached alignment). This provides strong evidence that the meadowfoam clones do encode LPAAT.

DNA sequence and translated amino acid sequence of meadowfoam LPAAT cDNA clones MELP2 and MELP4 are provided in Figures 17 and 18. The translated amino acid sequence of Melp2 demonstrates approximately 63% amino acid sequence identity to the coconut LPAAT (105 differences in 281 amino acids). A number of amino acid regions of 100% sequence identity spanning at least 6 contiguous amino acids are discovered by comparison of the complete encoding sequences for the coconut and meadowfoam LPAAT. These regions include LLPWPY, GNLYGH, RIDRSNP, KNLSLI, LPIVPM, FPEGTRS, GRLLPFKKGF, LTGTHLAWRK, and PITVKY. These amino acid sequences may be used to prepare additional probes and/or PCR primers for isolation of LPAAT encoding sequences from additional plant species.

To facilitate construct preparation, the coding region of LPAAT cDNA clone MELP2 is PCR amplified using the following oligonucleotide primers:

5867 5' CAUCAUCAUCAUGTCGACAATGGCCAAAACTAGAACTAGCT 3'
5868 5' CAUCAUCAUCAUGTCGACGGATCCTCACTTTGAGCGATTTGTGCT 3'.

Primer 5867 introduces a *Sal*I cloning site immediately upstream of the ATG translation start codon and primer 5868 introduces *Bam*HI and *Sal*I cloning sites immediately 3' to the translation stop codon of the MELP2 cDNA. The PCR product is cloned into pAMP1 (BRL/GIBCO) to yield pCGN7685 and sequenced to verify that mutations were not introduced by PCR.

Example 10 Expression of LPAAT in *E. coli*

An LPAAT clone may be expressed in *E. coli* to provide a convenient source of the protein for antibody production and for confirmation of expression of LPAAT activity. For example, the coconut LPAAT cDNA insert from pCGN5503 (COLP4) is mutagenized by PCR to insert a *Sal*I restriction site immediately upstream of the ATG start codon at nucleotides 259-261 of the sequence shown in Figure 13, and a *Bam*HI site immediately downstream of the TAA stop codon at nucleotides 1183-1185 of the sequence shown in Figure 13. The LPAAT encoding sequence is cloned as a *Sal*I/*Bam*HI fragment into a commercial cloning vector, CloneAmp (BRL), and the resulting construct is designated pCGN5504.

The LPAAT encoding region in pCGN5504 is transferred as a *Sal*I/*Bam*HI fragment into *E. coli* expression vector pCGN7645 resulting in pCGN5505 for expression of LPAAT from a T7 promoter. pCGN7645 was constructed by cloning a synthetic oligonucleotide linker containing a Shine-Delgarno sequence and *Sal*I, *Bam*HI and *Pst*I restriction sites into *Xba*I/*Bam*HI digested pET3A (Rosenberg *et al.* (1987) *Gene 56*:125-135). The sequence of the oligonucleotide linker is as follows:

5' CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGGTCGACGGATCCCTGCAGATC 3'.

E. coli BL21(DE3) cells containing the LPAAT construct pCGN5505 are grown at 37°C, pelleted and resuspended in 50 mM HEPES, 1 M NaCl, 10 mM EDTA, 100 µM Pefabloc (Boehringer Mannheim), 1 µM leupeptin, 0.1 µM pepstatin A, 5 mM ß-mercaptoethanol, pH 7.5, and were broken by sonication. The samples are centrifuged at 12,000*g* for 15 minutes. The resulting supernatant fractions are centrifuged for two hours at 134,000*g*, and the pelleted membranes suspended in 50 mM HEPES, 200 mM NaCl, 20% (w/v) glycerol, 5 mM ß-mercaptoethanol, pH 7.5. Membrane fractions are assayed for acyl-CoA substrate specificities with 12:0-LPA and various acyl-CoA species as described in Example 1. Membrane preparations from cultures of *E. coli* and from immature coconut endosperm are combined with soybean phospholipids and diluted in 1M NaCl in Buffer A to simulate the pre-assay conditions of the solubilized enzyme.

Cells expressing the coconut cDNA showed higher activity on medium-chain substrates, especially 12:0-CoA, than control *E. coli*, which preferred 18:1-CoA. The LPAAT activity of the *E. coli* expressed enzyme was also shown to be specific for acyl-CoA vs. acyl-ACP substrates. The coconut LPAAT was most active with 10:0-, 12:0- and 14:0-CoA substrates, with 18:0-CoA being less well utilized and with some minor activity detectable on 8:0-CoA. When the *E. coli* background is subtracted from pCGN5505 cultures, the resulting profile is very similar to that obtained from membrane fractions of immature coconut endosperm.

The techniques described above may also be used for expression of a meadowfoam clone. Full length clones isolated from a library in accordance with Example 8B can be directly assayed for activity because the LPAAT is expressed as a fusion protein with lacZ or alternatively expressed in an LPAAT deficient E. coli strain such as described by Coleman (*Mol. Gen. Genet.*(1992) *232*:295-303).

Example 11 Constructs for Plant Transformation

DNA constructs for use in plant transformation are prepared. For uses in expression in plant oilseed crops for modification of TAG, LPAAT encoding sequences may be inserted into expression cassettes containing regulatory regions which provide for preferential expression in plant seed tissues. Examples of genes from which such expression cassettes may be prepared include seed ACP, a Bce4 gene from *Brassica* seeds, and a *Brassica* napin gene. See, for example, Kridl *et al.* (in *Control of Plant Gene Expression* (1993) Chapter 30, pages 481-498, ed. D.P.S. Verma, CRC Press) for a discussion expression cassettes for use in expression of genes in plant seed tissues.

A. Napin Expression Constructs

A napin expression cassette, pCGN1808, which may be used for expression of wax synthase or reductase gene constructs is described in Kridl *et al.* (*Seed Science Research* (1991) *1*:209-219), which is incorporated herein by reference.

Alternatively, pCGN1808 may be modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, *supra*). Synthetic oligonucleotides containing *Kpn*I, *Not*I and *Hin*dIII restriction sites are annealed and ligated at the unique *Hin*dIII site of pCGN1808, such that only one *Hin*dIII site is recovered. The resulting plasmid, pCGN3200 contains unique *Hin*dIII, *Not*I and *Kpn*I restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *Hin*dIII and *Sac*I and ligation to *Hin*dIII and *Sac*I digested pIC19R (Marsh, *et al.* (1984) *Gene 32*:481-485) to make pCGN3212. The extreme 5'-sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *Sac*I site and the junction of the napin 5'-

promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains *Cla*I, *Hin*dIII, *Not*I, and *Kpn*I restiction sites as well as nucleotides 408-423 of the napin 5'-sequence (from the *Eco*RV site) and the reverse primer contains the complement to napin sequences 718-739 which include the unique *Sac*I site in the 5'-promoter. The PCR was performed using a Perkin Elmer/Cetus thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene 19*:259-268) and digested with *Hinc*II to give pCGN3217. Sequence of pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The resulting expression cassette pCGN3221, is digested with *Hin*dIII and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hin*dIII. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *Hin*dIII, *Not*I and *Kpn*I restriction sites and unique *Sal*I, *Bgl*II, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions and may be used to insert the LPAAT gene of interest.

For example, the *Sal*I/*Bam*HI fragment of pCGN5504 containing the entire coconut LPAAT encoding region is ligated into *Sal*I/*Bgl*II digested pCGN3223 to provide an expression construct pCGN5509 having the coconut LPAAT encoding sequence positioned for transcription of the sense sequence under regulation of the napin promoter.

For expression of meadowfoam LPAAT in plant seeds, pCGN7685 was digested with *Sal*I and *Bam*HI and the resulting LPAAT encoding fragment was cloned into pCGN3223 digested with *Sal*I and *Bgl*II to yield pCGN7692.

B. Oleosin Expression Constructs

A cassette for cloning of sequences for transcription under the control of 5' and 3' regions from an oleosin gene may be prepared as follows. Sequence of a *Brassica napus* oleosin gene was reported by Lee and Huang (*Plant Phys.* (1991) *96*:1395-1397). Primers to the published sequence are used in PCR reactions to obtain the 5' and 3' regulatory regions of an oleosin gene from *Brassica napus* cv. Westar. Two PCR reactions were performed, one to amplify approximately 950 nucleotides immediatley upstream of the ATG start codon for the oleosin gene, and one to PCR amplify approximately 600 bp including and downstream of the TAA stop codon for the oleosin gene. The PCR products were cloned into plasmid vector pAMP1 (BRL) according to manufacturer's protocols to yield plasmids pCGN7629 which contains the oleosin 5' flanking region and pCGN7630 which contains the 3' flanking region. The PCR primers included convenient restriction sites for cloning the 5' and 3' flanking regions together into an expression cassette. A *Pst*I fragment containing the 5' flanking region from pCGN7629 was cloned into *Pst*I digested pCGN7630 to yield plasmid pCGN7634. The *Bss*HII (New England BioLabs) fragment from pCGN7634, which contains the entire oleosin expression cassette was cloned into *Bss*HII digested pBCSK+ (Stratagene) to provide the oleosin cassette as plasmid pCGN7636. The oleosin cassette is flanked by *Bss*HII, *Kpn*I and *Xba*I restriction sites, and contains *Sal*I, *Bam*HI and *Pst*I sites for insertion of DNA sequences of interest between the 5' and 3' oleosin regions.

For example, the *Sal*I/*Bam*HI fragment of pCGN5504 containing the entire LPAAT encoding region is ligated into *Sal*I/*Bam*HI digested pCGN7636 to provide an expression construct pCGN5508 having the coconut LPAAT encoding sequence positioned for transcription of the sense sequence under regulation of the oleosin promoter.

C. Binary Constructs for Plant Agrobacterium-Mediated Plant Transformation

Constructs for plant transformation are prepared by transfer of the expression cassettes containing LPAAT sequences into convenient cloning sites on a binary vector such as those described by McBride *et al.* (*supra*).

Additional binary vectors are prepared from pCGN1578, pCGN1559 and other vectors described by McBride *et al.* (supra) by substitution of the pCGN1578 and pCGN1559 linker regions with a linker region containing the following restriction digestion sites:*Asp*718/*Asc*I/*Pac*I/*Xba*I/*Bam*HI/*Swa*I/*Sse*8387 (*Pst*I)/*Hin*dIII. This results in pCGN1578PASS or pCGN1559PASS, and other modified vectors which are designated similarly. *Asc*I, *Pac*I, *Swa*I and *Sse*8387 have 8-base restriction recognition sites. These enzymes are available from New England BioLabs: *Asc*I, *Pac*I; Boehringer Manheim: *Swa*I and Takara (Japan): *Sse*8387.

The binary constructs are then transformed into cells of an appropriate *Agrobacterium* strain, such as EHA101 (Hood *et al.* (1986) *J. Bacteriol. 168*:1291-1301) as per the method of Holsters *et al.* (*Mol. Gen. Genet.* (1978) *163*:181-187) for use in preparation of transgenic plants.

A binary construct for transformation with the napin 5'/coconut LPAAT/napin 3' construct is prepared by cloning the approximately 3.9 kb *Hin*dIII fragment of pCGN5509 into *Hin*dIII digested pCGN1578PASS resulting in pCGN5511.

A binary construct for transformation with the oleosin 5'/coconut LPAAT/oleosin 3' construct is prepared by cloning the approximately 2.6 kb *Bss*HII fragment of pCGN5508 into *Asc*I digested pCGN1578 resulting in pCGN5510.

A binary construct for transformation with the napin 5'/meadowfoam LPAAT/napin 3' construct is prepared by cloning the *Hin*dIII fragment of pCGN7692 containing the napin/meadowfoam LPAAT gene fusion into *Hin*dIII digested pCGN1559PASS to yield pCGN7695.

Example 12 Transformation with LPAAT Constructs

A variety of methods have been developed to insert a DNA sequence of interest into the genome of a plant host to obtain the transcription or transcription and translation of the sequence to effect phenotypic changes.

Transgenic *Brassica* plants (variety 212/86 or low linolenic varieties, such as , for example) are obtained by *Agrobacterium*-mediated transformation as described by Radke *et al.* (*Theor. Appl. Genet.* (1988) *75*:685-694; *Plant Cell Reports* (1992) *11*:499-505). Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) *85*:5536-5540). Other plant species may be similarly transformed using related techniques. When triglycerides containing very long chain fatty acids are of interest, use of high erucic acid rapeseed (HEAR) varieties will be particularly useful. An example of such a HEAR oil variety is Resten.

Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology 10*:286-291) may also be used to obtain nuclear transformed plants comprising the viral single subunit RNA polymerase expression constructs described herein.

For modification of TAG by incorporation of medium-chain fatty acids into the *sn*-2 position, transformation of plants containing significant levels of medium-chain fatty acids is desired. Such plants may be obtained by transformation with acyl-ACP thioesterases having preferential activity on medium-chain fatty acyl-ACP. (See WO 92/20236 and WO 94/10288.)

For modification of TAG by incorporation of very long chain fatty acids into the *sn*-2 position, transformation of plants having significant levels of very long chain fatty acids is desired. Such plants include high erucic acid rapeseed (HEAR) varieties or plants which have been transformed with a very long chain acyl-CoA synthase which provides for production of very long chain fatty acids.

(See co-pending USSN 08/265,047 filed 6/23/94 and PCT US94/13686 filed 11/30/94.)

Example 13 Analysis of Transgenic Plants

Seeds from transgenic plants containing the LPAAT constructs are assayed for LPAAT activity as described in Example 1. Plants identified as positive for LPAAT expression may be out-crossed to plants containing high levels of the desired fatty acids. For example, if a tri-laurin oil is desired, one may cross the LPAAT expressing plants with a plant having high levels of C12 fatty acids. Increased C12 levels may be produced as the result of expression of a C12 preferring acyl-ACP thioesterase from California bay (WO 92/20236 and WO 94/10288). In this manner, a ready source of C12 acyl-CoA donor substrate for LPAAT activity is provided.

A. LPAAT Activity in Transgenic Plants

Membrane fractions are prepared from immature seeds of transgenic plants containing constructs for seed expression of LPAAT as follows. Approximately 0.5-1 g (fresh weight) of immature seeds are ground in an ice-cold mortar with 5 ml extraction buffer comprising 0.1M HEPES-HCl pH 7.5, 3M NaCl, 10mM DIECA, 0.1mM Pefabloc, 1µM leupeptin, 1µM pepstatin 'A' (last 4 ingredients added just before use). A small amount of sand may be included for grinding. The sample is then centrifuged at 10,000 rpm for approximately 50 minutes.

The fat pad resulting from centrifugation is discarded, and the supernatant fraction is re-centrifuged at 36,000 rpm for 2 hours. The resulting pellet is resuspended in 250µl "P2" buffer (50mM HEPES-HCl pH 7.5, 1M NaCl, 20% v/v glycerol, 5mM 2-mercaptoethanol (ME)). Resuspended P2 preparations are either assayed immediately or frozen in liquid nitrogen for storage at -70°C and later assay.

If previously frozen, P2 preparation are re-homogenized and kept on ice. Into each glass assay vial is added 50µl of "5x" assay buffer (0.5M HEPES-HCl pH 7.5, 25%

v/v glycerol, 50mM EDTA, 10µl 5M NaCl, 122µl H_2O, 2.5µl 2mM 12:0-LPA or 18:1-LPA) and 5µl acyl-radiolabeled (see Example 1) 12:0-CoA or 18:1-CoA respectively. Reactions are started by addition of 50µl of a diluted P2 preparation sample.

For initial assays, a 10-fold P2 dilution was used, and the reaction was allowed to run for 30 minutes at 30°C. Reactions are stopped by addition of 250µl 1M KCl in 0.2M H_3PO_4. The following are then added to the reaction mixture: 40µl 1mg/ml BSA, 750µl $CHCl_3/CH_3OH$ (2/1 v/v), and 50µg unlabeled phosphatidic acid carrier (1 mg/ml in $CHCl_3/CH_3OH$, 2/1 v/v). (The phosphatidic acid ideally has 12:0 or 18:1 acyl groups according to the substrates used in the assay, but satisfactory results may also be obtained if they are different.) The samples are mixed thoroughly and briefly centrifuged to separate layers. The top layer is discarded, and 100µl samples of of the bottom layer are applied to TLC plates (Silica "G") or placed in scintillation vials. For scintillation counting the solvent is evaporated with a warm air stream prior to addition of scintillant.

The TLC is conducted with chloroform/pyridine/formic acid (50/30/7 or 50/25/7, v/v) as the ascending solvent. Radioactive zones are visualized and quantitated on the dried plates using a radiochromatogram scanner (Ambis Inc.). Phosphatidate product radioactivity on TLC is expressed as a percentage of total lane radioactivity, and this ratio is then used to calculate actual product radioactivity from the scintillation counting data.

Results of these assays are presented in Table 4 below.

TABLE 4

12:0/18:1 LPAAT Activity Ratios in Transgenic *Brassica*

Plant line	Activity Ratio	Plant line	Activity Ratio
Control-1	0.48	5511- 1	2.8
Control-2	0.68	5511- 2	1.8
Control-3	0.79	5511- 3	2.6
Control-4	0.93	5511- 4	0.84
Control-5	0.50	5511- 5	2.4
		5511- 6	2.2
		5511- 7	2.5
		5511- 8	2.4
		5511- 9	2.8
		5511-10	2.6

The assays for LPAAT activity were conducted with the substrate combinations 12:0-LPA + 12:0-CoA, and 18:1-LPA + 18:1-CoA. Both types of activity were shown to depend on the presence of the LPA substrate. Activity with the 12:0 substrate pair was expressed relative to activity with the 18:1 substrate pair. This ratio was typically 0.5 for control seed (no introduced coconut LPAAT gene), and substantially higher (often > 2.0) for seeds of plants transformed with the coconut LPAAT gene. The increase in this ratio is as an indication of increased preference of LPAAT activity for 12:0 substrates over 18:1 substrates and therefore of the expression of the coconut 12:0-preferring LPAAT activity in the transgenic plants.

Further investigation of this assay for comparison of activities on 12:0 *versus* 18:1 substrates showed that there was little dependence of the measured activities on the concentration of "P2" preparation. While useful for identifying a difference in substrate preference of control and transformant preparations, the assay did not distinguish seeds having high medium-chain LPAAT activity from those with lower levels of activity because the reaction ran to completion in much less than the 30 minute

incubation period. The time-course and preparation-dependence of the assay were examined in detail and the assay modified as follows. The P2 preparation was diluted 20-fold and 40-fold, and the reaction time was shortened to 10 minutes. The reaction is approximately linear with respect to time and enzyme concentration for 10 minutes at activities not exceeding 1500 cpm, and thus the results from the dilution which provides 1500 or <1500 cpm PA product (TLC-corrected scintillation count) are taken as representing LPAAT activity. P2 preparations were thawed and re-assayed under these conditions, using only the 12:0 substrates to compare medium-chain LPAAT enzyme activities in transgenic plants on a fresh-weight basis. Results of these assays are presented in Table 5 below.

TABLE 5

LPAAT Activity (12:0-CoA and 12:0-LPA Substrates)

Event	Mean Acty. (cpm PA product)	Diln. (fold)	Acty. x Diln. (cpm)	Seed Fresh Wt. (g)	Corrected Acty/wt. (cpm/g)
5511-1	2651*	40	106040*	0.67	158.3*
5511-2	1306	20	22120	0.73	35.8
5511-4	331	20	6620	0.23	28.8
5511-5	1191	20	23820	0.56	42.5
5511-7	1834	20	36680	0.91	40.3
5511-3	2240*	40	89600*	0.75	119.5*
5511-6	1447	20	28940	0.60	48.2
5511-8	1905	40	76200	0.65	117.2
5511-9	3227*	40	129080*	0.79	163.4*
5511-10	1379	40	55160	0.65	84.9

* Still offscale in assay

The above results demonstrate that transgenic *Brassica* plants having varying levels of activity provided by expressed coconut medium-chain LPAAT may be obtained.

B. Substrate Specificity of LPAAT Activity

Detailed acyl-CoA chain-length specificity analysis of control P2 preparations and LPAAT-transformed line 5511-5 were determined as described above, using the original 30 minute reaction time. The acyl-CoA substrates for this analysis are present in methanol, and thus the assay also had the following modifications. The first solution placed in the assay vial was the volume of each radiolabeled acyl-CoA required for 5μM final assay concentration. The methanol was evaporated with a nitrogen stream, and the remaining assay components are added as 215μl of a prepared mixture comprising 1250μl "5x" buffer, 250μl 5M NaCl, 63μl 2mM 12:0-LPA, 3825μl H_2O, and 25μl crude soybean phospholipids as a 125mg/ml solution in 2-methoxyethanol. After mixing to redissolve the acyl-CoA off the vial wall, the reaction is started by addition of 25μl of a 5-fold diluted P2 preparation and continued as before. Acyl-CoA substrates used in this analysis were 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, and 18:1. The acceptor substrate in these assays was 12:0-LPA. Results of these assays are presented in Table 6 below.

TABLE 6

LPAAT Acyl-CoA Specificities in Transgenic Rapeseed

	Cpm PA Product	
Donor Substrate	5511-5	Control
8:0	454	149
10:0	5648	831
12:0	9337	1758
14:0	5080	1544
16:0	4569	3034
18:0	2038	1514
18:1	8430	7946

The specificity profile for transformant 5511-5 is as expected for coconut medium-chain LPAAT activity superimposed on the control rapeseed LPAAT activity.

C. Breeding to Combine Medium-Chain LPAAT and Medium-Chain Acyl-ACP Thioesterase Genes

To produce transgenic *Brassica* seeds containing TAG having significant levels of 12:0 acyl groups incorporated at the *sn*-2 position, crosses are performed between the above CGNE5511 transgenic plants (female) and transgenic *Brassica* plants expressing a bay 12:0 acyl-ACP thioesterase (male) as the result of transformation with pCGN3828 (napin 5'/bay thioesterase/napin 3'; see Wo 92/20236). Seeds from the pCGN3828 plants typically contain approximately 50% laurate in the seed oil, primarily at the *sn*-1 and *sn*-3 positions.

D. Analysis of *sn*-2 Fatty Acyl Composition of TAG

To identify effects of the expressed LPAAT on the fatty acid compositions of transgenic seed oils, the fatty acid composition of extracted oils is determined by acid methanolysis as described by Browse *et al.* (*Anal. Biochem.* (1986) *152*:141-145). In addition, analysis of individual triglyceride types, for example, to determine percentage of tri-laurin or tri-erucin triglycerides, may be conducted by HPLC resolution as described by Jeffrey *et al.* (*JAOCS* (1991) *68*:289-293) or Nikolova-Damyanova *et al.* (*JAOCS* (1990) *67*:503-507).

Analyses of the acyl compositions of the sn-2 and sn-1+3 positions of TAG may be conducted using the lipase digestion protocol (Brockerhoff (1975) *Meth. Enzymol.* *35*:315-325). Ideally with this protocol, the lipase cleaves fatty acids from the sn-1 and sn-3 positions, and not from the sn-2 position. Thus, the fatty acids in the resulting mono-glyceride are presumed to be those in the sn-2 position. However, it is noted that those previously attempting to study TAG having shorter-chain fatty acids by this method (Entressangles *et al.* (1964) *Biochim. Biophys. Acta* *84*:140-148), reported that shorter-chain fatty acids located at the sn-2 position were quickly hydrolyzed during such a digestion, which the authors reported to be the result of a spontaneous migration of internal shorter-chain fatty acids towards outer positions in diglycerides and monoglycerides.

Thus, oil distilled from mature transgenic and control seeds is subjected to a lipase digestion protocol modified from Brockerhoff *et al.* (*supra*), to minimize acyl migration. This distinguishes acyl compositions of the sn-2 and sn-1+3 combined positions. The modifications are briefly as follows: pH is lowered to neutrality, reaction time is shortened, samples are maintained at acidic pH thereafter, and digestion products are chromatographed on borate-impregnated TLC plants. The chromatographed products are then eluted and analyzed as fatty acid methyl esters as before. In this manner the percentage of fatty acids, such as medium-chain C12 or C14 fatty acids or long-chain C22:1 fatty acids in the *sn*-2 position is determined. The modified procedure was verified using steochemically defined structured TAGs and is conducted as follows.

Generally in the lipase procedure, only positive-displacement pipetors are used as oil and organic solvents cannot be delivered reliably by negative-displacement pipetors. Additionally, care should be taken when evaporating solvents to bring the sample only barely to dryness. When C10 or shorter acyl groups are present avoid dryness altogether. Plasticware or kitchen glassware that can contribute fatty acid contamination should be avoided. Glassware may be pre-rinsed with chloroform/methanol 2/1 (v/v) if necessary.

In 15-ml screw-cap (teflon liner) vial combine 2 ml 0.1M Tris-HCl, pH 7.0, 0.2 ml 2.2% w/v $CaCl_2$, 0.5 ml 0.05% w/v bile salts (Sigma), and 10 µl (10 µg if solid) oil or TAG sample. Sonicate briefly in a sonication bath to disperse at least some of the oil. The suspension should develop a cloudy appearance after a few minutes.

Prepare lipase dilution using an active suspension of lipase, such as *Rhizopus arrhizus* lipase (Sigma, L4384) and hold on ice (4°C). (Activity will be lost if suspension is frozen). Enzyme batches may be checked by testing various dilutions of the suspension with water in the overall procedure, using oil containing unsaturated fatty acids and visualizing the extent of digestion by System 1 TLC (see below) with iodine staining. The correct dilution should

result in approximately 50% digestion of the TAG. (Further digestion risks increasing attack on the MAG product.) Typically dilution of the Sigma *Rhizopus arrhizus* lipase suspension with water to about 600,000 units/ml gives an appropriate concentration.

Each reaction is run individually. Add 100 µl of the water-diluted lipase to start the reaction, cap the vial, and immediately start a continuous vortex mixing for 1.5 minutes. Make and break the vortex several times during this mixing so as to prevent stratification. A white ppt must form during the 1.5 min "incubation". The precipitate comprises calcium salts of released fatty acids, and is an indication that the reaction is proceeding.

At the end of the 1.5 min mixing incubation, stop the reaction by adding 0.5 ml 6M HCl and mixing briefly. Immediately add 2.6 ml chloroform/methanol 2/1 v/v, shake well and place in ice while the other lipase digestions are performed. Note that the white ppt will now completely redissolve.

Remove all the vials from ice, mix well once again, and spin briefly to sharpen the layers. The digestion products are in the lower layer. Using a Pasteur pipet remove the lower layer to a new 15-ml vial. Re-extract the original digestion mixture with 1.6 ml straight chloroform, mix well, spin, and combine this lower layer with the previously removed one. The combined lower, organic layers are blown to near-dryness under N_2 and just enough heat to prevent the samples from getting very cold.

The TLC plates for acyl migration are 500 µm preparative Sil-G pre-loaded with boric acid and containing no fluorescent indicator. The pre-loading is carried out by ascending migration of 5% w/v boric acid in 1/1 v/v acetonitrile/methanol for at least 90 minutes. The plated are dried and stored at room temperature until ready for use. Heating "activation" may be necessary in damp climates.

Two solvent systems are suitable, both ascending the plates for exactly 1 hour even if the solvent doesn't reach

the top of the plate, as longer runs result in reduced resolution due to the extreme volatility of the solvents.

System 1 - n-hexane/diethyl ether/acetic acid, 70/30/1 v/v
System 2 - Diethyl ether/acetic acid, 100/1 v/v

System 1 is used to evaluate and monitor the lipase reaction, as it allows recovery of TAG, DAG, fatty acid, and MAG. System 2 may be used for routine use and yields the best purity of the MAG product required for the *sn*-2 determination.

Prior to spotting the plates, score down the middle with a pencil so that two samples can be applied (left and right). (Sample chromatography is performed in the same direction as the borate loading.) Also remove 0.5 cm of layer from each side to eliminate edge effects, and draw a line 2 cm up from the bottom as a loading guide. Redissolve each dried sample in 100 µl chloroform/methanol 2/1 (v/v) and apply along the loading line on the half-plate. Rinse the vial twice with 100 µl chloroform/methanol 2/1 (v/v) each time and load over the top of the sample. Air-dry the loading area and run the solvent. Let plates air-dry in hood.

To ensure minimal acyl rearrangements for *sn*-1 and *sn*-3 analyses of the products, the procedure should be conducted without interruption from the start of the lipase reaction.

The TLC plates are visualized with Rhodamine spray, ~1% w/v Rhodamine 6G in acetone. The plates are sprayed until they are an overall medium-pink color, allowed to dry a few minutes, and viewed under UV light. Lipids fluoresce yellow on an orange background. Desired zones are outlined in pencil. When using system 2, MAG zone is routinely 50-75% of the distance up the plate and the rest of the products are at the top. The MAG area may appear multi-zoned due to some chain-length resolution, but should be outlined for excision as a single overall zone.

The zones are scraped onto clean paper and transferred to large screw-cap (teflon liner) test tubes. Add 10 ml

chloroform/methanol 2/1 (v/v), shake, and let stand for at least an hour. Filter through Whatman paper directly into 100-ml rotary evaporation flasks. Rinse the tubes twice through the filters with 5 ml chloroform/methanol 2/1 (v/v) each time. (The Rhodamine dye will co-elute with the lipids and will track with them through the procedure until the final hexane extraction of fatty acid methyl esters (FAMES), when it will be left behind.) Rotary-evaporate at room temperature or up to 30°C, to reduce volume to about 100 µl. Transfer to 15-ml screw-cap vial, along with a couple of 100 µl chloroform/methanol 2/1 (v/v) rinses of the flask, and blow down to near-dryness under N2.

To the nearly dry samples add 2 ml freshly-prepared 5% (w/v) sulfuric acid in methanol. Relatively new methanol which has not had a chance to absorb much water should be used. Also add to the samples 1 ml of toluene containing desired internal standard at 0.5 mg/ml TAG (*e.g.* tri-17:0 etc.). Incubate at 90°C for 2 hours, tightening the caps after the first 2 minutes and again after about 15 minutes. After the vials have cooled, add 2 ml 0.9% w/v NaCl and 0.5 ml n-hexane. Mix thoroughly, let stand a few minutess to separate layers, and sample the top layer into the g.c. vial. Fatty acid composition is determined by analysis for fatty acid methyl esters (FAME) as described by Browse *et al.* (*Anal. Biochem.* (1986) *152*:141-145).

The composition of the MAG zone is taken as the composition at *sn*-2 of the original oil or TAG sample. The average composition at the primary (*sn*-1 and -3) positions is computed using the formula (3TAG-MAG)/2 on the % of each acyl group. (Example: an oil containing 50 mol % 12:0 overall and 5 mol % 12:0 at *sn*-2 has an average of 145/2 = 72.5 mol % 12:0 at the primary positions.)

The following data (Table 7A) were obtained with hexane-extracted oil of 20 (pooled) mature seeds resulting from crosses of the indicated 5511 transformed *Brassica napus* plants with pollen from pCGN3828 transformed *Brassica napus* plants.

TABLE 7A

sn-2 Fatty Acyl Composition of Triacylglycerols

LPAAT Parent	LPAAT acty. of parent (cpm/q)	Mol % 12:0 Overall	Mol % 12:0 2-MAG
5511-4	28.8	35.0	9.1
5511-6	48.2	32.6	7.4
5511-8	117.2	39.0	24.8

TABLE 7B

sn-2 Fatty Acyl Analysis of Control Samples

		Mol % 12:0 at:		
Sample	*sn*-2 expected	*sn*-2 measured	*sn*-1,3 avg. (calculated)	Overall
OLaO (*sn*)	100	96.3	-	-
	100	96.3	-	-
(S)LaOO	0	2.5	-	-
	0	4.2	-	-
(R)LaOO	0	2.6	-	-
	0	2.1	-	-
Line 23-198 oil	-	5.2	71.8	49.6
	-	5.5	71.7	-

(O = 18:1, La = 12:0, "R" configuration has the unique acyl at *sn*-1, "S" configuration has the unique acyl at *sn*-3)

The above data demonstrate that 12:0 is incorporated into the *sn*-2 position as the result of the medium-chain LPAAT activity. The procedure typically gives a value of 2-5 mol % for the laurate-producing line 23-198 (no introduced LPAAT) or for standard TAG having no 12:0 at *sn*-2 due to the imperfect specificity of the lipase. The

high level of 12:0 at *sn*-2 in the 5511-8 segregating seeds is consistent with the high enzyme activity of the LPAAT parent relative to the parents of the 5511-4 and 5511-6 crosses, and with altered 18:2 and 18:3 contents of the oil observed in half-seed fatty acid analyses of the 5511-8 seeds. The average laurate content of the oil in these F1 seed is lower than that of the BTE-expressing parent due to segregation of the BTE gene as a result of the crosses.

Seeds resulting from crosses between other 5511 transgenic plants and plants expressing medium-chain thioesterase enzymes may result in a range of levels of medium-chain fatty acids in the *sn*-2 position. For example, transgenic plant 5511-1, 5511-3 and 5511-9 have increased medium-chain LPAAT activity as compared 5511-8, and thus may lead to transgenic plant seeds having higher proportions of medium-chain fatty acids in the *sn*-2 position.

Furthermore, for increased production of C14 fatty acids in transgenic plant seeds, the medium-chain LPAAT expressing plants may be crossed with plants expressing acyl-ACP thioesterases providing for increased production of C14 fatty acids in plant seeds. Such plants and constructs which may be used to produce such plants are described in co-pending application USSN 08/383,756 filed on February 2, 1995.

In the above examples, solubilization and properties of LPAAT activity from plant seed tissues are described. A protocol is provided to obtain substantially purified medium-chain acyl-CoA-preferring LPAAT from coconut endosperm. Various properties of the protein are described, including methods to obtain and use amino acid and nucleic acid sequences related thereto. Nucleic acid and amino acid sequences corresponding to a coconut and a meadowfoam LPAAT protein are provided, and constructs for expression of LPAAT in host cells are described. Thus, through this invention, one can obtain the amino acid and nucleic acid sequences which encode LPAATs from a variety of sources and for a variety of applications. These LPAAT sequences may

then be expressed in transgenic plants to obtain altered triacylglycerides as described.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

What is claimed is:

1. A plant 1-acylglycerol-3-phosphate acyltransferase characterized as:

(i) being free from cytoplasmic membranes of said plant;

(ii) having preferential activity toward an acyl-CoA donor substrate as compared to an acyl-ACP donor substrate; and

(iii) having an amino acid sequence comprising the following peptides: (a) FPEGTRS, (b) GRLLPFKKGF, (c) LTGTHLAW and (d) PITVKY.

2. The acyltransferase of Claim 1 further characterized as:

(i) capable of catalyzing the production of 1,2-dilauroylglycerol-3-phosphate from 1-lauroylglycerol-3-phosphate and lauroyl-CoA; and

(ii) having preferential activity toward a lauroyl-CoA substrate as compared to a lauroyl-ACP substrate.

3. The acyltransferase of Claim 2 wherein said plant is coconut.

4. The acyltransferase of Claim 1 wherein said plant is meadowfoam.

5. A DNA construct comprising a first DNA sequence encoding a plant 1-acylglycerol-3-phosphate acyltransferase peptide joined to a second DNA sequence heterologous to said first DNA sequence, wherein said first DNA sequence is obtainable by:

(A) an amplification reaction comprising the steps of:

(i) contacting, under polymerase chain reaction conditions, (a) an oligonucleotide encoding six contiguous amino acids of the peptide represented as SEQ ID NO: 1 and (b) DNA from a plant 1-acylglycerol-3-phosphate acyltransferase source;

(ii) recovering a DNA sequence which encodes a plant 1-acylglycerol-3-phosphate acyltransferase peptide; or

(B) screening a plant gene library with a nucleic acid probe encoding at least 5 consecutive amino acids of a

plant 1-acylglycerol-3-phosphate acyltranferase peptide selected from the group consisting of LLPWPY, GNLYGH, RIDRSNP, KNLSLI, LPIVPM, FPEGTRS, GRLLPFKKGF, LTGTHLAWRK, and PITVKY.

6. The DNA construct of Claim 5 wherein said six contiguous amino acids of the peptide represented as SEQ ID NO: 1 are FPEGTR.

7. The DNA construct of Claim 5 wherein said first DNA sequence is capable of amplification by a combination of primers wherein the 5'-> 3' primer encodes FPEGTRS and the 3'->5' primer is 5'RACEamp.

8. The DNA construct of Claim 5 comprising a sequence encoding at least 8 consecutive amino acids of a plant 1-acylglycerol-3-phosphate acyltransferase protein.

9. The DNA construct of Claim 5 wherein a DNA sequence encoding an active plant 1-acylglycerol-3-phosphate acyltransferase is recovered.

10. The DNA construct of Claim 5 wherein said plant is coconut, and wherein said 1-acylglycerol-3-phosphate acyltransferase source is immature endosperm.

11. The DNA construct of Claim 5 wherein said plant is meadowfoam and said 1-acylglycerol-3-phosphate acyltransferase source is immature embryo.

12. A chimeric gene comprising DNA sequences in the 5' to 3' direction of transcription, transcriptional and translational regulatory initiation regions functional in a host cell, a DNA sequence encoding a plant 1-acylglycerol-3-phosphate acyltransferase, and a transcription termination regulatory region functional in said cell, wherein at least one of said regulatory regions is heterologous to said DNA sequence or at least one of said DNA sequences is heterologous to said cell, and wherein said plant 1-acylglycerol-3-phosphate acyltransferase encoding sequence is obtainable by:

(A) an amplification reaction comprising the steps of:

(i) contacting, under polymerase chain reaction conditions, (a) an oligonucleotide encoding six contiguous amino acids of the peptide represented as SEQ ID NO: 1 and

(b) DNA from a plant 1-acylglycerol-3-phosphate acyltransferase source;

(ii) recovering a DNA sequence which encodes a plant 1-acylglycerol-3-phosphate acyltransferase peptide; and

(iii) probing a cDNA or genomic DNA library isolated from a plant 1-acylglycerol-3-phosphate acyltransferase source with the DNA sequence recovered in (ii); or

(B) screening a plant gene library with a nucleic acid probe comprising at least 15 consecutive nucleotides of the acyltransferase encoding sequence shown in any one of Figures 10-18,

wherein said probing or said screening is conducted under conditions whereby a DNA sequence encoding an active plant 1-acylglycerol-3-phosphate acyltransferase is recovered.

13. The chimeric gene of Claim 12 wherein said six contiguous amino acids of the peptide represented as SEQ ID NO: 1 are FPEGTR.

14. The chimeric gene of Claim 12 wherein said first DNA sequence is capable of amplification by a combination of primers wherein the 5'-> 3' primer encodes FPEGTRS and the 3'->5' primer is 5'RACEamp.

15. The chimeric gene of Claim 12 comprises a sequence encoding at least 8 consecutive amino acids of a plant 1-acylglycerol-3-phosphate acyltransferase protein.

16. The chimeric gene of Claim 12 wherein said plant is meadowfoam and said 1-acylglycerol-3-phosphate acyltransferase source is immature embryo.

17. The chimeric gene of Claim 12 wherein said host cell is a plant cell.

18. The chimeric gene of Claim 17, wherein said transcriptional initiation region is from a gene preferentially expressed in plant seed tissue.

19. The chimeric gene of Claim 12, wherein said plant acyltransferase source is selected from the group consisting of coconut endosperm tissue and meadowfoam embryo tissue.

20. The chimeric gene of Claim 12, wherein said plant 1-acylglycerol-3-phosphate acyltransferase protein is

preferentially active towards medium chain as compared to longer chain acyl-CoA substrates.

21. The chimeric gene of Claim 12 wherein said acyltransferase sequence is in an antisense orientation.

22. A cell comprising a construct according to Claim 12.

23. A plant cell comprising a construct according to Claim 17.

24. A plant comprising a plant cell of Claim 23.

25. A plant of Claim 24, wherein said plant is a *Brassica* plant.

26. A method of producing a plant 1-acylglycerol-3-phosphate acyltransferase in a cell comprising

transforming a cell with a DNA construct of Claim 12, and

growing said cell to produce quantities of said plant 1-acylglycerol-3-phosphate acyltransferase.

27. A method of modifying the fatty acyl composition of triglycerides in a plant seed comprising,

growing a plant to seed, wherein said plant contains a DNA construct providing for expression of a foreign plant 1-acylglycerol-3-phosphate acyltransferase protein in seeds of said plant.

28. The method of Claim 27 wherein said plant further comprises a DNA construct providing for expression of a medium-chain preferring acyl-ACP thioesterase in seeds of said plant.

29. The method of Claim 27 wherein said medium-chain acyl-ACP thioesterase demonstrates preferential activity towards C12 acyl-ACP substrates.

30. The method of Claim 28 wherein said plant seed comprises an increased proportion of medium-chain fatty acyl groups in the triglyceride *sn*-2 position as compared to the proportion of medium-chain fatty acids in the triglyceride *sn*-2 position of control plants, wherein said control plants comprise a DNA construct for expression of a medium-chain acyl-ACP thioesterase in seeds of said plant, and wherein said control plants lack a 1-acylglycerol-3-phosphate acyltransferase protein having preferential activity on medium-chain fatty acids.

31. The method of Claim 30 wherein the proportion of medium-chain fatty acids in the triglyceride *sn*-2 position in said plant seeds is at least 10%.

32. The method of Claim 30 wherein the proportion of medium-chain fatty acids in the triglyceride *sn*-2 position in said plant seeds is at least 20%.

33. The method of Claim 30 wherein the proportion of medium-chain fatty acids in the triglyceride *sn*-2 position in said plant seeds is at least 25%.

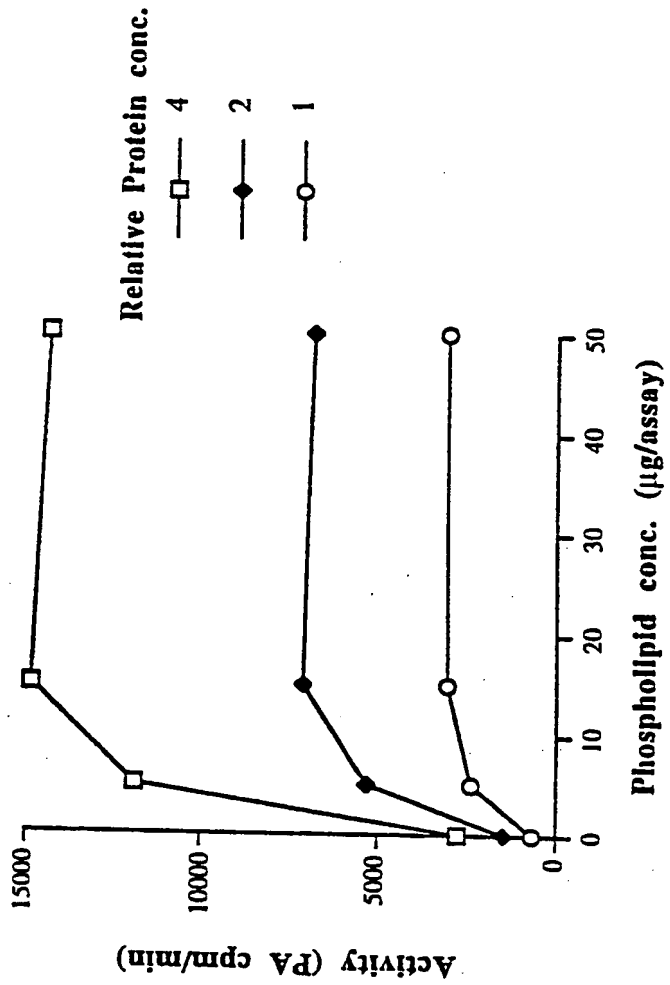

Relative Protein conc.
4
2
1
Activity (PA cpm/min)
15000
10000
5000
0
0
10
20
30
40
50
Phospholipid conc. (µg/assay)

FIGURE 1

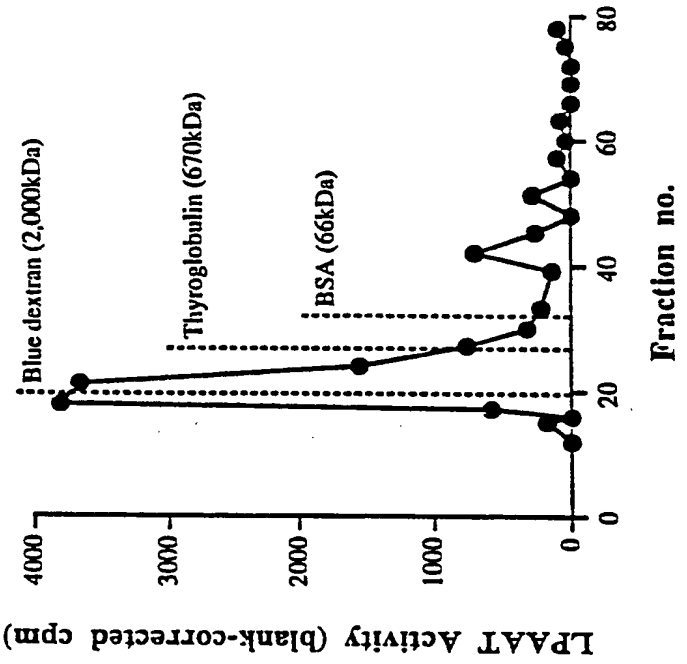

LPAAT Activity (blank-corrected cpm)
4000
3000
2000
1000
0
0
20
40
60
80
Fraction no.
Blue dextran (2,000kDa)
Thyroglobulin (670kDa)
BSA (66kDa)

FIGURE 2

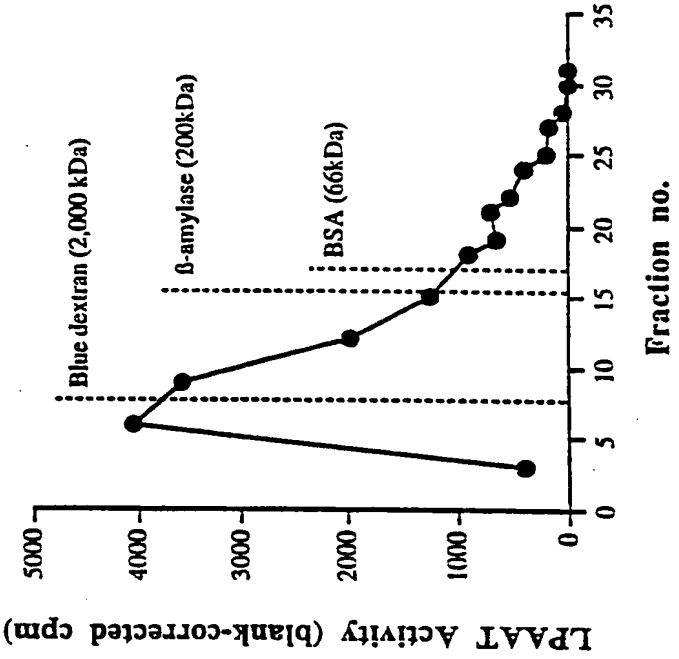

LPAAT Activity (blank-corrected cpm)
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
35
Fraction no.
Blue dextran (2,000 kDa)
β-amylase (200kDa)
BSA (66kDa)

FIGURE 3

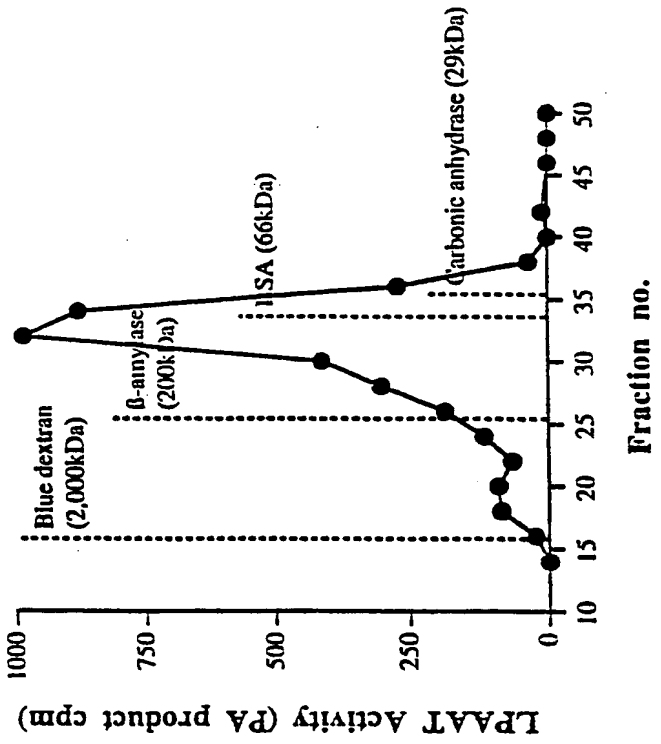

LPAAT Activity (PA product cpm)
1000
750
500
250
0
10
15
20
25
30
35
40
45
50
Fraction no.
Blue dextran (2,000kDa)
β-amylase (200kDa)
BSA (66kDa)
Carbonic anhydrase (29kDa)

FIGURE 4

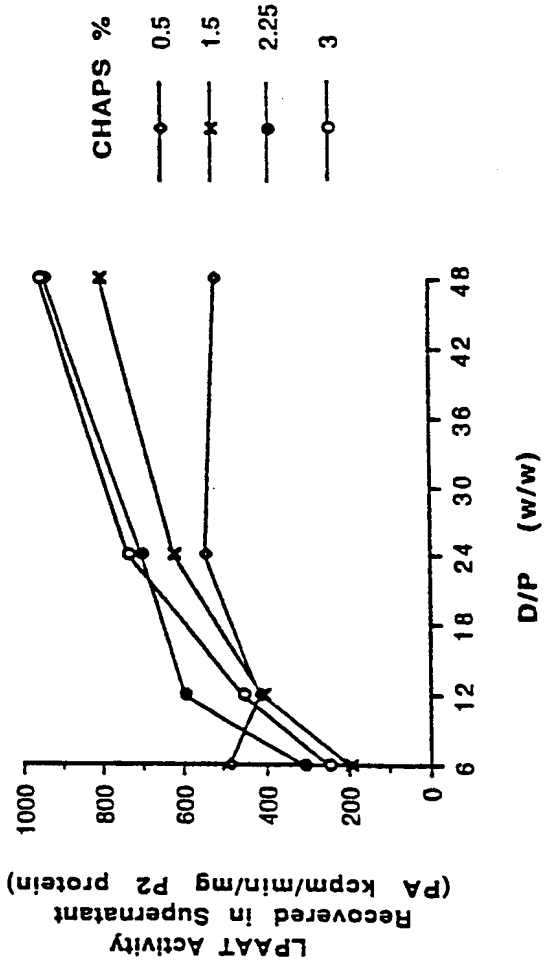

CHAPS %
0.5
1.5
2.25
3
LPAAT Activity
Recovered in Supernatant
(PA kcpm/min/mg P2 protein)
1000
800
600
400
200
0
6
12
18
24
30
36
42
48
D/P (w/w)

FIGURE 5

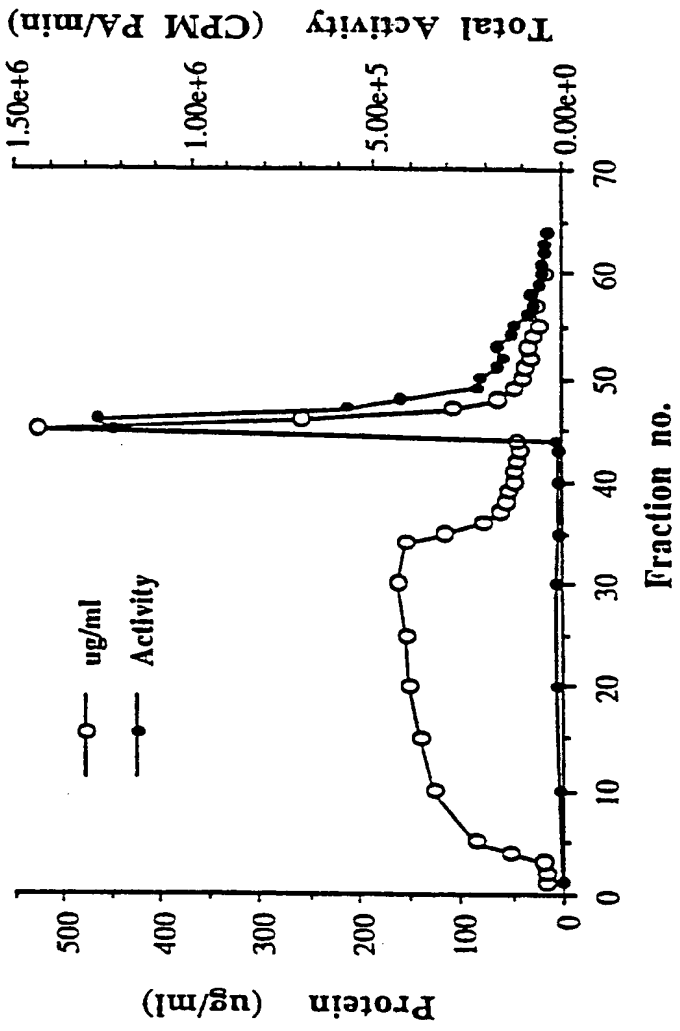

Total Activity (CPM PA/min)
1.50e+6
1.00e+6
5.00e+5
0.00e+0
Protein (ug/ml)
500
400
300
200
100
0
ug/ml
Activity
0
10
20
30
40
50
60
70
Fraction no.

FIGURE 6

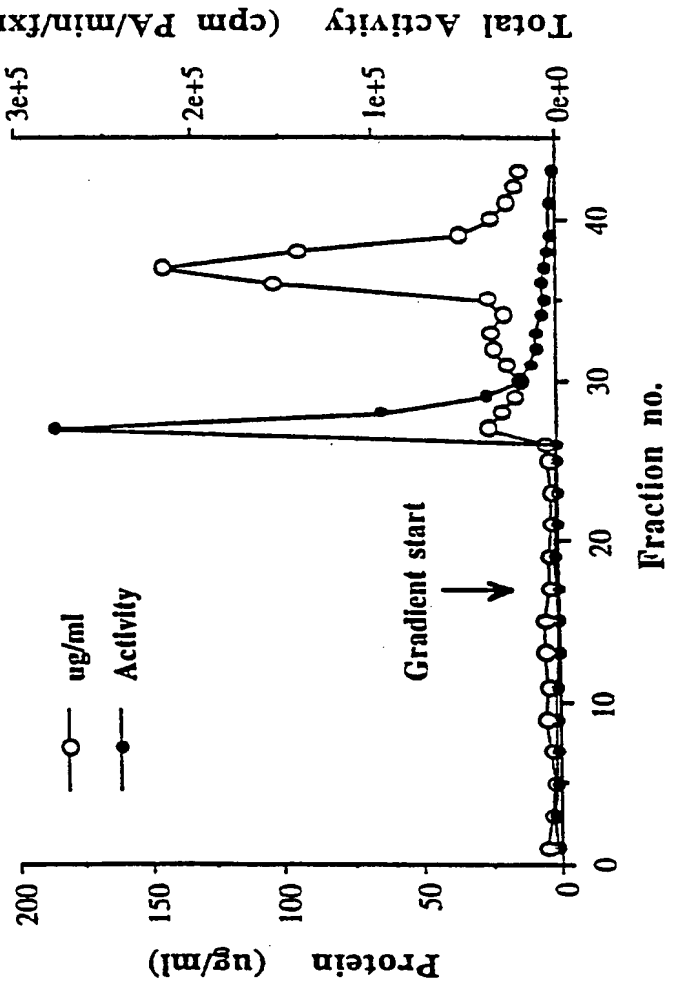

Total Activity (cpm PA/min/fxn)
3e+5
2e+5
1e+5
0e+0
Protein (ug/ml)
200
150
100
50
0
ug/ml
Activity
Gradient start
0
10
20
30
40
Fraction no.

FIGURE 7

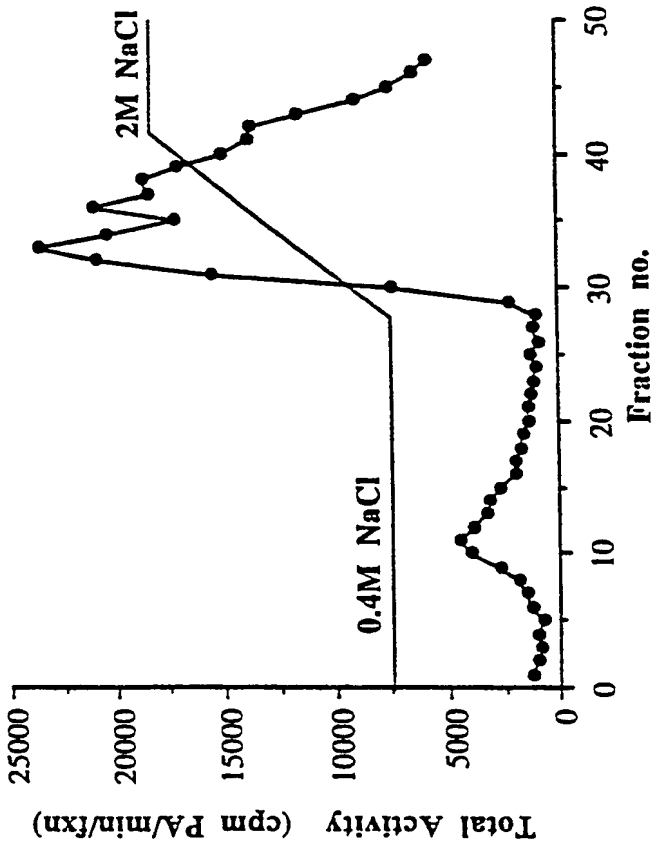

2M NaCl
0.4M NaCl
Total Activity (cpm PA/min/fxn)
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
Fraction no.

FIGURE 8

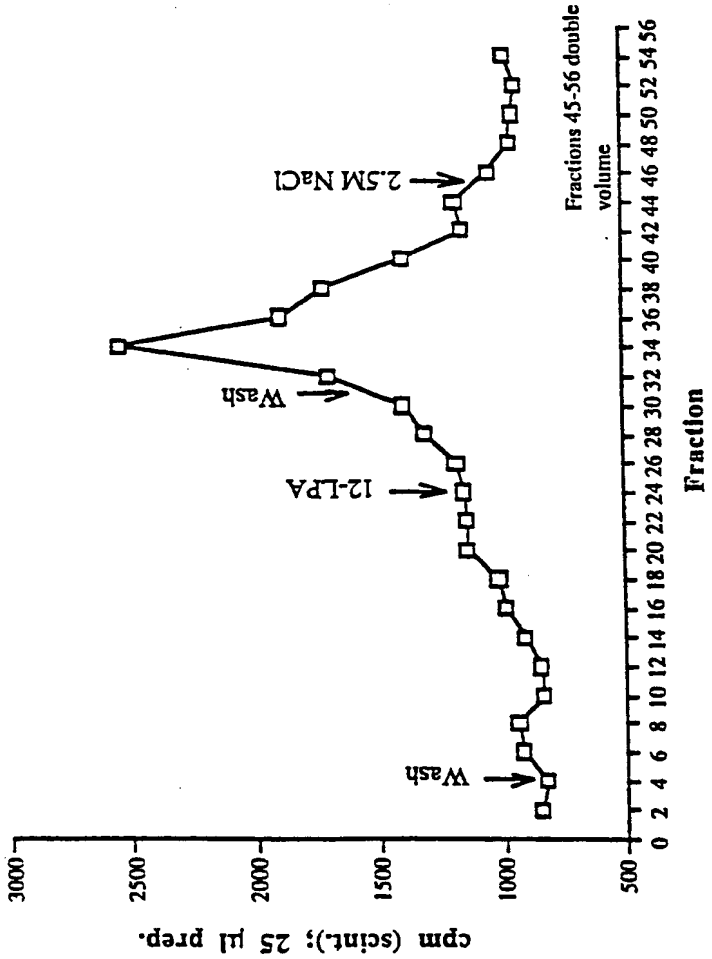

cpm (scint.); 25 µl prep.
3000
2500
2000
1500
1000
500
Wash
12-LPA
Wash
2.5M NaCl
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56
Fraction
Fractions 45-56 double volume

FIGURE 9

```
CTACTACTAC TA ATA ATA TTT CCG GAG GGG T ACT CGA TCG AAA ACA GGA    49
              Ile Ile Phe Pro Glu Gly   Thr Arg Ser Lys Thr Gly
                          SQ1256

AGG CTG CTT CCA TTT AAG AAG GGT TTT ATT CAC ATA GCA CTT CAG ACA    97
Arg Leu Leu Pro Phe Lys Lys Gly Phe Ile His Ile Ala Leu Gln Thr

CGG TTG CCG ATA GTT CCA ATG GTG CTG ACG GGT ACC CAT CTA GCT TGG   145
Arg Leu Pro Ile Val Pro Met Val Leu Thr Gly Thr His Leu Ala Trp
                                    SQ1271

AGG AAG AAC AGT TTG CGA GTC AGA CCA GCA CCT ATC ACA GTG AAA       190
Arg Lys Asn Ser Leu Arg Val Arg Pro Ala Pro Ile Thr Val Lys
                    SQ1276 and SQ1282

TAC TTC TCA CCC ATA AAA ACT GAT GAC TGG GAA GAA GAA AAG ATC       235
Tyr Phe Ser Pro Ile Lys Thr Asp Asp Trp Glu Glu Glu Lys Ile
     SQ1262 and SQ1281

AAT CAT TAT GTG GAA ATG ATC CAC ATGATGATGA TG                    271
Asn His Tyr Val Glu Met Ile His
               SQ1272
```

FIGURE 10

```
CTACTACTAC TA ATA ATA TTC CCC GAA GGT ACT CGA TCG AAA ACA GGA AGG    51
              Ile Ile Phe Pro Glu Gly Thr Arg Ser Lys Thr Gly Arg
                                  SQ1256

CTG CTT CCA TTT AAG AAG GTAACGATCA TAACATGCGT GTATATTTGT             99
Leu Leu Pro Phe Lys Lys

ATGTTTATCC ATTTTATTCT TCTGCTTGTG CTTCTCGTTT CTTCATTTTC TGTTGCAG     157

GGT TTT ATT CAC ATA GCA CCT CAG ACA CGG TTG CCG ATA GTT CCA ATG     205
Gly Phe Ile His Ile Ala Pro Gln Thr Arg Leu Pro Ile Val Pro Met

GTG CTG ACG GGT ACC CAT CTA GCT TGG AGG AAG AAC AGT TTG CGA GTC     253
Val Leu Thr Gly Thr His Leu Ala Trp Arg Lys Asn Ser Leu Arg Val
                  SQ1271

AGA CCA GCA CCT ATC ACA GTG AAA  TAC TTC TCA CCC ATA AAA ACT GAT    301
Arg Pro Ala Pro Ile Thr Val Lys  Tyr Phe Ser Pro Ile Lys Thr Asp
      SQ1276 and SQ1282                  SQ1262 and SQ1281

GAC TGG GAA GAA GAA AAG ATC AAT CAT TAT GTC GAA ATG ATT CAC         346
Asp Trp Glu Glu Glu Lys Ile Asn His Tyr Val Glu Met Ile His
                                        SQ1272

ATGATGATGA TG                                                       358
```

FIGURE 11

TACTACTACT A ATA ATA TTT CCC GAG GGT ACT CGA TCG AAA ACA GGA AGG 50
Ile Ile Phe Pro Glu Gly Thr Arg Ser Lys Thr Gly Arg
SQ1256

CTG CTT CCA TTT AAG AAG GGT TTT ATT CAC ATA GCA CTT CAG ACA CGG 98
Leu Leu Pro Phe Lys Lys Gly Phe Ile His Ile Ala Leu Gln Thr Arg

TTG CCG ATA GTT CCA ATG GTG CTG ACG GGT ACC CAT CTA GCT TGG AGG 146
Leu Pro Ile Val Pro Met Val Leu Thr Gly Thr His Leu Ala Trp Arg
SQ1271

AAG AAC AGT TTG CGA GTC AGA CCA GCA CCT ATC ACA GTG AAA TAC TTT 194
Lys Asn Ser Leu Arg Val Arg Pro Ala Pro Ile Thr Val Lys Tyr Phe
SQ1276 and SQ1282

TCG CCG ATC AAA ATGATGATGA TG 218
Ser Pro Ile Lys
SQ1262

FIGURE 12

```
CGGCAGACCC CTCTCTTCTT AGAAACCACC CGTCAGTATT TCTTAATTTT CTTTACTCTT   60

TTTCTCTATT TGGTCTGCAC TCTAGAATCT TCTCTTTCTT CTCTCTCCAC CAAGAACCCA  120

TAGAATTTGT TCGTTGCTGG ATTCCGATTC CGACCTATTC GCCAGTTCCC TACTCGGAAC  180

CCTCAACCCT TTACGTAGTC CTCGTTTGCC TTTCTTGCTC GTGGTATTGG TGGTGGGAAG  240

TGGGGGATAT ATAGTCCT ATG GAT GCT TCA GGG GCA AGT TCG TTC TTG CGG     291
                   Met Asp Ala Ser Gly Ala Ser Ser Phe Leu Arg

GGC CGT TGT CTG GAG AGC TGC TTC AAA GCG AGC TTC GGG ATG TCC CAA     339
Gly Arg Cys Leu Glu Ser Cys Phe Lys Ala Ser Phe Gly Met Ser Gln

CCG AAA GAT GCA GCC GGG CAA CCG AGT CGC CGG CCG GCC GAC GCG GAT     387
Pro Lys Asp Ala Ala Gly Gln Pro Ser Arg Arg Pro Ala Asp Ala Asp

GAC TTT GTG GAT GAT GAT AGA TGG ATT ACT GTC ATC CTG TCG GTC GTT     435
Asp Phe Val Asp Asp Asp Arg Trp Ile Thr Val Ile Leu Ser Val Val
```

FIG. 13A

```
AGG ATC GCT GCT TGC TTT CTG TCG ATG ATG GTT ACC ACC ATC GTG TGG    483
Arg Ile Ala Ala Cys Phe Leu Ser Met Met Val Thr Thr Ile Val Trp

AAC ATG ATC ATG CTG ATT TTG CTC CCT TGG CCA TAT GCT CGG ATC AGG    531
Asn Met Ile Met Leu Ile Leu Leu Pro Trp Pro Tyr Ala Arg Ile Arg

CAG GGA AAC TTG TAT GGC CAT GTT ACC GGG CGG ATG CTG ATG TGG ATC    579
Gln Gly Asn Leu Tyr Gly His Val Thr Gly Arg Met Leu Met Trp Ile

TTA GGG AAC CCA ATA ACA ATA GAA GGT TCT GAA TTC TCG AAC ACA AGG    627
Leu Gly Asn Pro Ile Thr Ile Glu Gly Ser Glu Phe Ser Asn Thr Arg

GCC ATC TAC ATC TGT AAT CAT GCA TCA CTT GTA GAC ATT TTT CTC ATC    675
Ala Ile Tyr Ile Cys Asn His Ala Ser Leu Val Asp Ile Phe Leu Ile

ATG TGG TTG ATT CCA AAG GGT ACC GTT ACC ATA GCA AAA AAA GAG ATC    723
Met Trp Leu Ile Pro Lys Gly Thr Val Thr Ile Ala Lys Lys Glu Ile
```

FIG. 13B

```
ATT TGG TAC CCA CTC TTT GGG CAG CTT TAT GTA TTG GCA AAC CAT CAG    771
Ile Trp Tyr Pro Leu Phe Gly Gln Leu Tyr Val Leu Ala Asn His Gln

CGA ATA GAC CGG TCC AAC CCA TCC GCT GCC ATT GAG TCA ATT AAA GAG    819
Arg Ile Asp Arg Ser Asn Pro Ser Ala Ala Ile Glu Ser Ile Lys Glu

GTA GCT CGA GCA GTT GTC AAG AAA AAC TTA TCG CTG ATC ATT TTT CCA    867
Val Ala Arg Ala Val Val Lys Lys Asn Leu Ser Leu Ile Ile Phe Pro

GAG GGT ACT CGA TCG AAA ACA GGA AGG CTG CTT CCA TTT AAG AAG GGT    915
Glu Gly Thr Arg Ser Lys Thr Gly Arg Leu Leu Pro Phe Lys Lys Gly

TTT ATT CAC ATA GCA CTT CAG ACA CGG TTG CCG ATA GTT CCA ATG GTG    963
Phe Ile His Ile Ala Leu Gln Thr Arg Leu Pro Ile Val Pro Met Val

CTG ACG GGT ACC CAT CTA GCT TGG AGG AAG AAC AGT TTG CGA GTC AGA   1011
Leu Thr Gly Thr His Leu Ala Trp Arg Lys Asn Ser Leu Arg Val Arg
```

FIG. 13C

```
CCA GCA CCT ATC ACA GTG AAA TAC TTC TCA CCC ATA AAA ACT GAT GAC   1059
Pro Ala Pro Ile Thr Val Lys Tyr Phe Ser Pro Ile Lys Thr Asp Asp

TGG GAA GAA GAA AAG ATC AAT CAT TAT GTG GAA ATG ATA CAT GCC TTG   1107
Trp Glu Glu Glu Lys Ile Asn His Tyr Val Glu Met Ile His Ala Leu

TAC GTG GAT CAC CTG CCG GAG TCT CAA AAA CCT TTG GTA TCA AAA GGG   1155
Tyr Val Asp His Leu Pro Glu Ser Gln Lys Pro Leu Val Ser Lys Gly

AGG GAT GCT AGC GGA AGG TCA AAT TCA TAAGTATAGG TTTCCTTGAG         1202
Arg Asp Ala Ser Gly Arg Ser Asn Ser

CATCATGTTG GTTATTATAT GCAGCAATAT GACAAGCATA AGTGTGACTT ATTTTAGAAA 1262

TATGTTCATG CCTTTTTTTT TTCCTTATCA GTACCATCAT GTGGAATAAA GAAACGCTTT 1322

NTGAAAAAAA AAAAAAAAAA AAAAAAAAAC TCGAGGGGGG GCCCGGTACC CAATTCGCCC 1382

TATAGTGAGT CGTATTACAA TCACTG                                      1408
```

FIG. 13D

Sequence Range: 1 to 517

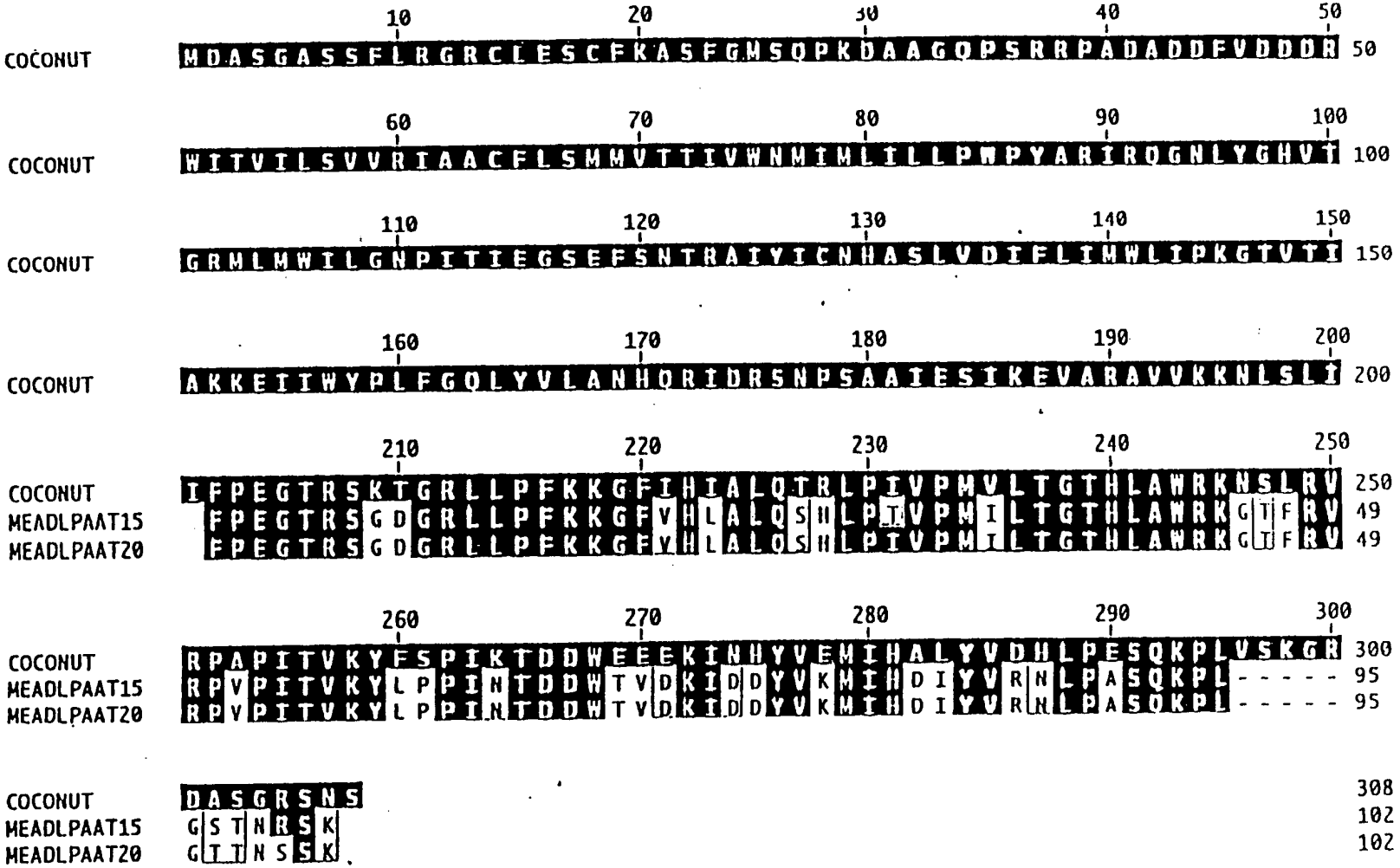
```
          10                  20                  30                  40
GAATTCAAGC TTC CCA GAG GGG ACC AGG TCG GGA GAT GGG CGT TTA
            F   P   E   G   T   R   S   G   D   G   R   L>

    50                  60                  70                  80                  90
CTT CCT TTC AAG AAG GGT TTT GTA CAT CTA GCA CTT CAG TCA CAC
 L   P   F   K   K   G   F   V   H   L   A   L   Q   S   H>

          100                 110                 120                 130
CTC CCG ACA GTT CCA ATG ATC CTT ACA GGT ACA CAT TTA GCA TGG
 L   P   T   V   P   M   I   L   T   G   T   H   L   A   W>

  140                 150                 160                 170                 180
AGG AAA GGT ACC TTC CGT GTC CGG CCA GTA CCC ATC ACT GTC AAG
 R   K   G   T   F   R   V   R   P   V   P   I   T   V   K>

          190                 200                 210                 220
TAC CTT CCT CCT ATA AAC ACT GAT GAT TGG ACT GTT GAC AAA ATT
 Y   L   P   P   I   N   T   D   D   W   T   V   D   K   I>

  230                 240                 250                 260                 270
GAC GAT TAC GTC AAA ATG ATA CAC GAC ATT TAT GTC CGC AAC CTA
 D   D   Y   V   K   M   I   H   D   I   Y   V   R   N   L>

          280                 290                 300                 310
CCT GCG TCT CAA AAG CCA CTT GGT AGC ACA AAT CGC TCA AAA TGA
 P   A   S   Q   K   P   L   G   S   T   N   R   S   K   *>

  320                 330                 340                 350                 360                 370
GTCGCTCTTGGCTCTAAACTTAGCAGAATGGATACGTACTTTTGTCTTGCTGCATGAAAA

  380                 390                 400                 410                 420                 430
GTTTAATCCTTTCTTGTGATATTAGATTATAGTGTAAGACTTTCATCTTAAAATAGTGTA

  440                 450                 460                 470                 480                 490
CCAGTACTTCTTGTTTGTAACCTTTACAATAAAAGTATGCCGTTGAAGAAAAAAAAAAAA

  500                 510
AAAAAAAAAGAGCTCCTGCAG
```

FIG. 14

Sequence Range: 1 to 508

```
          10                  20                  30                  40
GAATTCAAGC TTT CCG GAG GGC ACA CGG TCG GGA GAT GGG CGT TTA
             F   P   E   G   T   R   S   G   D   G   R   L>

    50                  60                  70                  80                  90
    *
CTT CCT TTC AAG AAG GGT TTT GTA CAT CTA GCA CTT CAG TCA CAC
 L   P   F   K   K   G   F   V   H   L   A   L   Q   S   H>

          100                 110                 120                 130
          *
CTC CCT ATA GTT CCG ATG ATC CTT ACA GGT ACA CAT TTA GCA TGG
 L   P   I   V   P   M   I   L   T   G   T   H   L   A   W>

  140                 150                 160                 170                 180
                      *
AGG AAA GGT ACC TTC CGT GTC CGG CCA GTA CCC ATC ACT GTC AAG
 R   K   G   T   F   R   V   R   P   V   P   I   T   V   K>

          190                 200                 210                 220
                              *
TAC CTT CCT CCT ATA AAC ACT GAT GAT TGG ACT GTT GAC AAA ATC
 Y   L   P   P   I   N   T   D   D   W   T   V   D   K   I>

  230                 240                 250                 260                 270
                                  *
GAC GAC TAC GTC AAA ATG ATA CAC GAC ATC TAT GTC CGC AAC CTA
 D   D   Y   V   K   M   I   H   D   I   Y   V   R   N   L>

          280                 290                 300                 310
                                          *
CCT GCG TCT CAA AAG CCA CTT GGT ACC ACA AAT AGC TCA AAG TGA
 P   A   S   Q   K   P   L   G   T   T   N   S   S   K   *>

  320                 330                 340                 350                 360                 370
                                              *
GTCGCTCTTGGCTCTAAAGTTAGCAGAATGGATACGTACTTTTGTCTTGCTGCATGAAAA

  380                 390                 400                 410                 420                 430
                              *
GTTTAATTCTTTCTTGTGATATTAGATTATAGTGTAAGACTTTCATCTTAAAATAGTGTA

  440                 450                 460                 470                 480                 490
                      *
CCAGTACTTCTTGTTTGTAACTTTTACAATAAAAGTATGCCGTTGAAGAAAAAAAAAGAA

  500
  *
GAGCTCCTGCAG
```

FIG. 15



DECORATION #1: SHADE (WITH SOLID BLACK) RESIDUES THAT MATCH COCONUT EXACTLY.

DECORATION #2: BOX RESIDUES THAT MATCH COCONUT WITHIN 2 DISTANCE UNITS.

FIG. 16

```
GAATTCGGCA CGAGAATCTC TCTTACTGGA TTTTAGGTCA AACAATCTCA TAGCCGGTTC   60

TATTC ATG GCC AAA ACT AGA ACT AGC TCT CTC CGC AAC AGG AGA CAA CTA  110
      Met Ala Lys Thr Arg Thr Ser Ser Leu Arg Asn Arg Arg Gln Leu

AAG ACG GCT GTA GCT GCT ACT GCT GAT GAT GAT AAA GAT GGG ATT TTT    158
Lys Thr Ala Val Ala Ala Thr Ala Asp Asp Asp Lys Asp Gly Ile Phe

ATG GTA TTG CTA TCG TGT TTC AAA ATT TTC GTT TGT TTT GCG ATA GTG    206
Met Val Leu Leu Ser Cys Phe Lys Ile Phe Val Cys Phe Ala Ile Val

TTG ATC ACG GCG GTG GCA TGG GGA CTA ATC ATG GTC TTG CTC TTA CCT    254
Leu Ile Thr Ala Val Ala Trp Gly Leu Ile Met Val Leu Leu Leu Pro

TGG CCT TAT ATG CGG ATT CGA CTA GGA AAT CTA TAC GGC CAT ATC ATT    302
Trp Pro Tyr Met Arg Ile Arg Leu Gly Asn Leu Tyr Gly His Ile Ile

GGT GGA TTA GTG ATA TGG CTT TAC GGA ATA CCA ATA GAG ATC CAA GGA    350
Gly Gly Leu Val Ile Trp Leu Tyr Gly Ile Pro Ile Glu Ile Gln Gly

TCT GAG CAT ACA AAG AAG AGG GCC ATT TAT ATA AGC AAT CAT GCA TCT    398
Ser Glu His Thr Lys Lys Arg Ala Ile Tyr Ile Ser Asn His Ala Ser
```


FIGURE 17A

```
CCT ATC GAT GCT TTT TTT GTT ATG TGG TTG GCT CCC ATA GGC ACA GTT    446
Pro Ile Asp Ala Phe Phe Val Met Trp Leu Ala Pro Ile Gly Thr Val

GGT GTT GCA AAG AAA GAG GTT ATA TGG TAT CCG CTA CTT GGA CAA CTA    494
Gly Val Ala Lys Lys Glu Val Ile Trp Tyr Pro Leu Leu Gly Gln Leu

TAT ACA TTA GCC CAT CAT ATT CGT ATA GAT CGG TCG AAC CCG GCT GCA    542
Tyr Thr Leu Ala His His Ile Arg Ile Asp Arg Ser Asn Pro Ala Ala

GCT ATT CAG TCT ATG AAA GAG GCA GTT CGT GTA ATA ACC GAA AAG AAT    590
Ala Ile Gln Ser Met Lys Glu Ala Val Arg Val Ile Thr Glu Lys Asn

CTC TCT CTG ATT ATG TTT CCA GAG GGA ACC AGG TCG GGA GAT GGC CGT    638
Leu Ser Leu Ile Met Phe Pro Glu Gly Thr Arg Ser Gly Asp Gly Arg

TTA CTT CCT TTC AAG AAG GGT TTT GTA CAT CTA GCA CTT CAG TCA CAC    686
Leu Leu Pro Phe Lys Lys Gly Phe Val His Leu Ala Leu Gln Ser His

CTC CCC ATA GTT CCG ATG ATC CTT ACA GGT ACA CAT TTA GCA TGG AGG    734
Leu Pro Ile Val Pro Met Ile Leu Thr Gly Thr His Leu Ala Trp Arg

AAA GGT ACC TTC CGT GTC CGG CCA GTA CCC ATC ACT GTC AAG TAC CTT    782
Lys Gly Thr Phe Arg Val Arg Pro Val Pro Ile Thr Val Lys Tyr Leu
```

FIGURE 17B

```
CCT CCT ATA AAC ACT GAT GAT TGG ACT GTT GAC AAA ATT GAC GAT TAC     830
Pro Pro Ile Asn Thr Asp Asp Trp Thr Val Asp Lys Ile Asp Asp Tyr

GTC AAA ATG ATA CAC GAC ATT TAT GTC CGC AAC CTA CCT GCG TCT CAA     878
Val Lys Met Ile His Asp Ile Tyr Val Arg Asn Leu Pro Ala Ser Gln

AAG CCA CTT GGT AGC ACA AAT CGC TCA AAG TGAGCCGCTC TTGGCTCTAA      928
Lys Pro Leu Gly Ser Thr Asn Arg Ser Lys

ACTTAGCAGA ATGGATACTT ACTTTTGTCT TGCTGCATGA AAAGTTTAAT CCTTTCTTGT  998

GATATTAGAT TGTAGTGTAA GACTTTCATC TTAAAATAGT GTACCAGTGC TTGTTGTTTG 1058

TAACTTTTAC AATAAAAGTA TGCCGTTGAA GAAGGGTGCA AGATTTAAAT AAAAATGAAT 1108

TCTATTTTTT CGATAAAAAA AAAAAAAAAA AACTCGAG                        1146
```

FIGURE 17C

```
GCCGGTTCTA TTC ATG GCC AAA ACT AGA ACT AGC TCT CTC CGC AAC AGG      49
               Met Ala Lys Thr Arg Thr Ser Ser Leu Arg Asn Arg

AGA CAA CTA AAG CCA GCT GTA ACT GCT ACT GCT GAT GAT GAT AAA GAT      97
Arg Gln Leu Lys Pro Ala Val Thr Ala Thr Ala Asp Asp Asp Lys Asp

GGG GTT TTT ATG GTA TTG CTA TCG TGT TTT AAA ATT TTC GTT TGT TTT     145
Gly Val Phe Met Val Leu Leu Ser Cys Phe Lys Ile Phe Val Cys Phe

GCG ATA GTT TTG ATC ACG GCG GTG GCA TGG GGA CTA ATC ATG GTC TTG     193
Ala Ile Val Leu Ile Thr Ala Val Ala Trp Gly Leu Ile Met Val Leu

CTC TTA CCT TGG CCT TAT ATG AGG ATA CGA CTA GGA AAT CTA TAC GGC     241
Leu Leu Pro Trp Pro Tyr Met Arg Ile Arg Leu Gly Asn Leu Tyr Gly

CAT ATC ATT GGT GGA TTA GTG ATA TGG CTT TAT GGA ATA CCA ATA GAG     289
His Ile Ile Gly Gly Leu Val Ile Trp Leu Tyr Gly Ile Pro Ile Glu

ATC CAA GGA TCT GAG CAT ACG AAG AAG AGG GCC ATT TAT ATA AGC AAT     337
Ile Gln Gly Ser Glu His Thr Lys Lys Arg Ala Ile Tyr Ile Ser Asn

CAT GCA TCT CCT ATC GAT GCT TTT TTT GTT ATG TGG TTG GCT CCC ATA     385
His Ala Ser Pro Ile Asp Ala Phe Phe Val Met Trp Leu Ala Pro Ile
```


FIGURE 18A

```
GGC ACA GTT GGT GTT GCA AAG AAA GAG GTT ATA TGG TAT CCG CTA CTT    433
Gly Thr Val Gly Val Ala Lys Lys Glu Val Ile Trp Tyr Pro Leu Leu

GGA CAA CTA TAT ACA TTA GCC CAT CAT ATT CGT ATA GAT CGG TCG AAC    481
Gly Gln Leu Tyr Thr Leu Ala His His Ile Arg Ile Asp Arg Ser Asn


CCG GCC GCG GCT ATT CAG TCT ATG AAA GAG GCA GTT CGT GTA ATA ACC    529
Pro Ala Ala Ala Ile Gln Ser Met Lys Glu Ala Val Arg Val Ile Thr


GAA AAG AAT CTC TCT CTG ATT ATG TTT CCA GAG GGA ACC AGG TCG GGA    577
Glu Lys Asn Leu Ser Leu Ile Met Phe Pro Glu Gly Thr Arg Ser Gly


GAT GGG CGT TTA CTT CCT TTC AAG AAG GGT TTT GTA CAT CTA GCA CTT    625
Asp Gly Arg Leu Leu Pro Phe Lys Lys Gly Phe Val His Leu Ala Leu


CAG TCA CAC CTC CCG ATA GTT CCA ATG ATC CTT ACA GGT ACA CAT TTA    673
Gln Ser His Leu Pro Ile Val Pro Met Ile Leu Thr Gly Thr His Leu


GCA TGG AGG AAA GGT ACC TTC CGT GTC CGG CCA GTA CCC ATC ACT GTC    721
Ala Trp Arg Lys Gly Thr Phe Arg Val Arg Pro Val Pro Ile Thr Val


AAG TAC CTT CCT CCT ATA AAC ACT GAT GAT TGG ACT GTT GAC AAA ATT    769
Lys Tyr Leu Pro Pro Ile Asn Thr Asp Asp Trp Thr Val Asp Lys Ile
```

FIGURE 18B

```
GAC GAT TAC GTC AAA ATG ATA CAC GAC ATT TAT GTC CGC AAC CTA CCT    817
Asp Asp Tyr Val Lys Met Ile His Asp Ile Tyr Val Arg Asn Leu Pro

GCG TCT CAA AAG CCA CTT GGT AGC ACA AAT CGC TCA AAA TGAGTCGCTC     866
Ala Ser Gln Lys Pro Leu Gly Ser Thr Asn Arg Ser Lys

TTGGCTCTAA ACTTAGCAGA ATGGATACGT ACTTTTGTCT TGCTGCATGA AAAGTTTAAT  926

CCTTTCTTGT GATATTAGAT TATAGTGTAA GACTTTCATC TTAAAATAGT GTACCAGTAC  986

TTCTTGTTTG TAACTTTTAC AATAAAAGTA TGCCGTTGAA GAAAAAAAAA AAAAAAAAA  1045
```


FIGURE 18C

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 95/03997

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 C12N9/10 A01H5/00 C11B1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,94 13814 (NICKERSON BIOCEM LTD ;SLABAS ANTONI RYSZARD (GB); BROWN ADRIAN PAU) 23 June 1994 see the whole document ---	5,12
A	PLANTA, vol. 185, no. 1, 1991 pages 124-131, HARES, W., ET AL. 'SUBSTRATE SPECIFICITIES OF THE MEMBRANE-BOUND AND PARTIALLY PURIFIED MICROSOMAL ACYL-COA:1-ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE FROM ETIOLATED SHOOTS OF PISUM SATIVUM (L.)' see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 1995	05.09.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE PHYTOCHEMICAL SOCIETY OF EUROPE, vol. 35, 1993 pages 81-95, SLABAS, A.R., ET AL. 'FATTY ACID SYNTHESIS IN OILSEEDS' see page 89, last paragraph - page 91, paragraph 1 ---	5
A	MOLECULAR AND GENERAL GENETICS, vol. 232, no. 2, March 1992 BERLIN DE, pages 295-303, COLEMAN, J., ET AL. 'CHARACTERIZATION OF THE ESCHERICHIA COLI GENE FOR 1-ACYL-SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE (PLSC)' see the whole document ---	5
A	PLANT PHYSIOLOGY, vol. 99, 1992 pages 1711-1715, LAURENT, P., ET AL. 'ORGAN- AND DEVELOPMENT-SPECIFIC ACYL COENZYME A LYSOPHOSPHATIDATE ACYLTRANSFERASES IN PALM AND MEADOWFOAM' see the whole document ---	1,4,11
A	FAT SCIENCE TECHNOLOGY, vol. 93, no. 8, August 1991 pages 288-290, WOLTER, F.P., ET AL. 'BIOCHEMICAL AND MOLECULAR BIOLOGICAL APPROACHES FOR CHANGING THE FATTY ACID COMPOSITION OF RAPE SEED OIL' see the whole document ---	1,4,5, 11,27
A	FAT SCIENCE TECHNOLOGY & 47TH ANNUAL MEETING OF THE GERMAN SOCIETY FOR FAT SCIENCE, 2-5 SEPTEMBER, 1991 , vol. 93, no. 11, November 1991 pages 417-418, PETEREK, G., ET AL. 'WEGE ZUR KLONIERUNG DER 1-ACYLGLYCERIN-3-PHOSPHAT-ACYLTRANSFERASE' see the whole document ---	1,4,5, 11,27
A	BIOL. CHEM. HOPPE SEYLER, & WORKSHOP HELD SEPTEMBER 10-13, 1991., vol. 372, no. 8, 1991 page 539 PETEREK, G., ET AL. 'APPROACHES OF CLONING THE I ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE' see the whole document --- -/--	1,4,5, 11,27

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 95/03997

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE EMBO JOURNAL, vol. 11, no. 13, 1992 pages 4685-4692, WOLTER, F.P., ET AL 'CHILLING SENSITIVITY OF ARABIDOPSIS THALIANA WITH GENETICALLY ENGINEERED MEMBRANE LIPIDS' see the whole document	12
A	WO,A,92 13082 (KIRIN BREWERY) 6 August 1992 see the whole document	12
A	WO,A,92 20236 (CALGENE INC) 26 November 1992 see page 37 - page 53	27-30

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INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 95/03997

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9413814	23-06-94	AU-B-	5656794	04-07-94
		CA-A-	2151147	23-06-94
WO-A-9213082	06-08-92	AU-A-	1163392	27-08-92
		EP-A-	0567648	03-11-93
		JP-T-	6504439	26-05-94
WO-A-9220236	26-11-92	EP-A-	0557469	01-09-93
		JP-T-	7501924	02-03-95